DEVELOPMENT OF AN EXPERIMENTAL MODEL TO INVESTIGATE THE COMMUNITY DYNAMICS OF SELECTED SAPROTROPHIC WOOD DECOMPOSER FUNGI FROM THE FRIGATE UNICORN

CRAIG J STURROCK

Ph.D. 2002

Development of an Experimental Model to Investigate the Community Dynamics of Selected Saprotrophic Wood Decomposer Fungi from the Frigate Unicorn

A thesis submitted in partial fulfilment of the

University of Abertay Dundee for the

Degree of Doctor of Philosophy

Craig John Sturrock

School of Science and Engineering

University of Abertay Dundee

March 2002

I certify that this thesis is a true and accurate version of the thesis approved by the examiners.

Signed [redacted]..... Date .20..May.2002.

(Director of Studies)

Declaration

I hereby declare that this thesis has been composed by myself and that it has not been accepted in any previous application for a degree. The work, of which it is a record, is my own, unless otherwise stated. All verbatims have been distinguished by quotation marks and sources of information specifically acknowledged by means of references.

20/05/02.

Craig J Sturrock

AKNOWLEDGEMENTS

I would like to express my sincere thanks to;

My supervisors Dr Nia White and Professor John Palfreyman for their valuable help, guidance and encouragement, Professor Karl Ritz for his additional advice and expertise, and the University of Abertay Dundee for their support throughout this project.

Dr Paul Birch, Dr David Cooke, and Dr James Duncan (Scottish Crop Research Institute) for all their help with the molecular biology work and Jim Bown, John Crawford, and Bill Samson (SIMBIOS, UAD) for their valuable help on the mathematical modelling aspect of this project. Daniel Cullen (USDA Forest Products Laboratory and Plant Pathology Department, University of Wisconsin, USA), Professor Lorna Casselton (Dept. Plant Sciences, University of Oxford) and Mimi Zolan (Indiana University, Bloomington, USA) for there advice and kind donation of the plasmid vectors used in the project.

Special thanks are also extended to the staff and students of the School and Science and Engineering, University of Abertay Dundee, for the help and friendship. Out-with the University, I am indebted to the love and support of my fiancée, Lisa, and all my family and friends in Dundee and beyond.

Dedicated to my Mother, Father, Nana.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

An experimental microcosm system developed to investigate the community dynamics of two- and three-fungal species interactions is described. The microcosm consists of tessellated nutrient agar tiles allowing the varied prescription of: (1) the number of interacting fungal species; (2) the spatial organisation (patchiness) of the distribution of individuals; and (3) the scale of the interaction arena. The system also allows the quantification of interaction outcome in terms of species occupancy within each tile using a destructive re-isolation based plating technique. The outcome and reproducibility of small-scale, pairwise confrontations were used to predict the behaviour of larger two- and three-species interactions. The influence of experimental factors such as species patch size and spatial distribution on the community dynamics were also investigated. The spatial heterogeneity displayed during large-scale three species tessellations was represented by a novel application of principal component analysis, which showed good intuitive agreement with visual assessment of the interaction outcome patterns. The development of a non-destructive method, based on green fluorescent protein labeling of one of the organisms studied, to continuously monitor the development of the interactions is also described.

Results indicated that for two species interactions of equal patch size the final outcomes of the large-scale tessellations could be extrapolated from the behaviour of relevant small-scale binary confrontations. However, further investigation revealed that species patch size influenced the temporal dynamics of the system. It was shown that larger patch sizes of species increased the time taken for one species to replace the other. Results also showed that

extrapolation of the behaviour of large-scale three species confrontations were not possible from combative hierarchy information derived from the outcome of binary tile confrontations. The outcomes of the three species interactions were shown to be neither random nor fully deterministic and that a degree of stochasticity was displayed in outcome for all tessellations. These findings suggest that the initial spatial distribution of species influence outcome and reproducibility of the interactions. The model therefore demonstrates the complex and coordinated behaviour of fungal mycelia on fungal community development.

The work to develop a non-destructive analysis system for the study of fungal interactions was unsuccessful. The work attempted to introduce the gene for green fluorescent protein into the genome of one of the study organisms, *C. marmorata,* via genetic transformation. Although, the introduction and expression of exogenous genetic material into the genome of *C. marmorata* was not successful, it was possible to isolate protoplasts from this species. This is the first known report of this for this species.

CHAPTER 1: INTRODUCTION

1.1. FUNGAL COMMUNITIES AND THEIR ROLE IN ECOSYSTEM FUNCTIONING

Fungi are eukaryotic organisms that display diversity both in form and function. Such diversity is epitomised by the vast number of fungal species that are found in terrestrial and aquatic habitats. Hawksworth (1991) estimated that 1.5 million species of fungi exist worldwide. However, this generous estimation has been the subject of much debate (see Hawksworth, 2000) with estimations both above and below this figure. These arguments are attributed to the difficulties associated in estimating the vast number of species occurring in tropical habitats, and associated with insect hosts.

In the natural environment fungi occur as assemblages of different species, thereby constituting a community. A salient feature of the fungal community is that frequent interactions will occur between fungi, other organisms and the physical environment. Knowledge of these interactions is therefore fundamental to understanding the structure and development of fungal communities and their role in ecosystem functioning (Rayner & Boddy, 1988; Christensen, 1989; Stahl & Christensen, 1992).

Possibly one of the most significant interactions that fungi have with their physical, abiotic environment is nutrient acquisition. Fungi are heterotrophic, obtaining the nutrients they require for growth from their surrounding environment by excretion of extracellular enzymes and/or absorption. A major distinction can be made between fungi based on their source of organic nutrients. Fungi may grow as either parasites on other living organisms or

as saprotrophs on non-living materials. Fungi may further be defined by whether their growth has a positive or negative effect on their host organism. For example, the plant pathogen *Phytophthora infestans,* the causal agent of potato blight, may be described as necrotrophic (disease feeding). However, many species form beneficial symbiotic relationships with their host species. Examples of such, are the association of fungal species with algae (lichens) or with plant roots (mycorrhizae) (Deacon, 2000).

Saprotrophic fungi (in combination with bacteria) are the principle decomposers of plant and animal detritus in the natural environment, thus recycling chemical elements back to the environment in a form other organisms may utilise. Fungi are ideally suited in this role. Their thread-like network of hyphae, that constitute the mycelium, can penetrate cells and other organic materials, secreting acids and powerful hydrolytic and/or oxidative enzymes that breakdown complex molecules into simpler components that are suitable for absorption. During this process carbon dioxide, nitrates and other inorganic compounds such as phosphorus, sulphur and iron may be released. The activity of saprotrophic fungi contributes to a major part of the carbon cycle. The carbon cycle is the reciprocal processes of photosynthesis and cellular respiration. Plant biomass is produced via the fixation of atmospheric carbon dioxide into organic molecules by photosynthesis. Plants are then either consumed or degraded releasing carbon dioxide into the atmosphere via cellular respiration. The role of saprotrophic fungi as decomposers can be highlighted by the vast quantities of organic matter they can degrade. It is estimated that approximately two tons of plant matter may be deposited per hectare of forest in temperate regions annually due to the accumulation leaf litter, fallen branches and even whole trees (Carlile and Watkinson, 1994). Therefore, without the degradative processes of fungi, life on earth would probably

come to an end due to the accumulation of plant matter and a lack of carbon dioxide for photosynthesis.

Although the importance of fungi in ecosystem functioning is widely acknowledged, the processes underlying the dynamics of fungal decomposer communities are poorly understood (Halley *et al*, 1994). For example, leaf litter decomposition is one of the most extensively studied and understood systems (Frankland, 1998, and references therein). Despite this, much of our knowledge consists of lists of successions of species rather than an understanding of the interacting processes taking place within the community. Furthermore, there is a growing awareness that environmental heterogeneity may have profound effects on the development of fungal communities (Ritz & Crawford, 1999). The majority of natural environments display both spatial and temporal heterogeneity and patchiness (Kotliar & Wiens, 1990; Levin, 1992; Wijesinghe & Hutchings, 1997) in terms of both abiotic and biotic factors. Therefore, the response of the fungal community to such heterogeneity is likely to be of significant importance in understanding the processes of decomposition in natural environments.

The aims of this chapter are:

- Introduce the structure and organisation of the fungal mycelium, and explain its significance in the development of fungal communities.
- Consider the ecology of fungi and the interaction of the mycelium with the abiotic and biotic environment.
- Discuss the approaches taken to study fungal communities and their current limitations.
- Outline the origins and development of the experimental basis of this study.

1.2. FUNGI, THEIR GROWTH AND MORPHOGENESIS

1.2.1. Major classifications of fungi

The kingdom *Fungi* has been divided into four phyla, *Chytridiomycota*, *Zygomycota*, *Ascomycota*, and *Basidiomycota* (Alexopoulous, *et al.,* 1996). In addition, there is also an informal group, the mitosporic fungi. This grouping is also known as Deuteromycota or the Imperfect fungi. The 'imperfection' that leads to the classification of mitosporic fungi is based on the absence of any known sexual stage in the life cycle in the majority of these species. One of the species investigated in this project (*Paecilomyces variotii*) belongs to the mitosporic fungi subdivision. There are many species that belong to this classification including plant pathogens such as *Fusarium* and *Verticillium*, the ubiquitous moulds *Penicillium* and *Aspergillus*, and also many species of soil fungi such as *Trichoderma* and *Cladosporium*. Mitosporic fungi reproduce asexually, via the production of spores termed conidia. The other two fungi (*Poria placenta* and *Coniophora marmorata*) investigated here belong to the phylum *Basidiomycota*. The name is derived from the name basidium, a 'club-like' cellular structure that forms four haploid spores during the diploid stage in the organism's life cycle. Mushrooms, bracket fungi, puffballs, and stinkhorns are all classified in the division Basidiomycota.

1.2.2. Hyphae

Hyphae are the basic unit of filamentous fungal growth and consist of apically extending protoplasmic tubes. As they grow and branch a network of filaments is produced which constitutes the mycelium. As opposed to determinate life forms, such as unicellular organisms and all animals which have genetically programmed limits in

both space and time, the growth of filamentous fungi is potentially unlimited (Rayner, 1994). Such indeterminate growth is of considerable importance for growth in a heterogeneous environment, as hyphae have the potential to extend great distances relative to their diameter, often spanning inhospitable regions, to locate new resource bases (Ritz, 1995). This property of hyphae is conferred by their polar growth. Polarised hyphal growth is achieved due to the 'plastic' properties of the hyphal apex which yield to the internal turgor pressure within the hypha (Robson, 1999). Growth is also mediated by the transport of vesicles to the hyphal apex, which provide the components for biosynthesis of the cell wall (Harold, 1997).

The structural integrity of hyphae is maintained via the fungal cell wall. In most filamentous fungi the cell wall consists of an inner wall composed of chitin microfibrils and an outer wall composed largely of β-1-3- and β-1-6- glucans (Robson, 1999). Melanins, branched polymers derived from aromatic amino acids, are also found in cell walls of many fungi (Cooke & Whipps, 1993). These compounds are highly resistant to enzymatic degradation and it is possible that melanins endow the hyphal wall with the capacity to resist lysis by other microorganisms (Carlile & Watkinson, 1994). Further, melanins may also protect fungi from damaging UV radiation and desiccation (Cooke & Whipps, 1993). Melanisation has also been proposed as a possible mechanism involved in cell wall insulation which is thought to be important during mycelial organisational events (Rayner, 1994). This concept will be given further discussion in 1.2.5.

The hyphae of higher fungi often possess frequent cross walls, know as septa, that divide the hyphae into compartments. Ultrastructural analyses have shown that septa

have several electron - dense layers perforated by a central pore (Hunsley & Gooday, 1974; Momany & Hamer, 1997). Septal ultrastructure has been found to vary depending on taxonomic grouping. For example, many deuteromycota and ascomycota tend to posses relatively simple septa (cross wall with a pore), however, the septa of basidiomycota are more complex, and are often termed 'dolipore septa'. Spherical structures called 'Woronin bodies' (Buller, 1933 (cited in Rayner & Boddy, 1988); Riechle & Alexander, 1965 (both cited in Rayner & Boddy, 1988)), are often seen associated with the central pore. The role of these Woronin bodies appears to be to plug the septal pore in older or damaged hyphae. It has been suggested that septa add rigidity to the hyphae, however, this is probably of secondary importance to the physiological role of septa which is to allow the fungus to compartmentalise differentiation and differentiate specialised cell types during mycelial organisation (Gull, 1978). Certain groups of fungi (e.g. Zygomycota) lack septa except to delimit reproductive structures or to 'seal off' damaged hyphae (Cooke & Rayner, 1984). Mycelia devoid of septa are termed coenocytic.

1.2.3. Mycelial differentiation and colonisation

Fungal colonization of a resource involves a sequential series of stages, classified by Rayner, Watling and Frankland (1985) as arrival, establishment, exploitation, and exit. Each of these stages involves the differentiation of the mycelium into specialized structures depending on local environmental stimuli.

1.2.3.1 Arrival modes

Fungal colonisation of a resource may be initiated by arrival of either a propagule (such as a spore) or as mycelium. Successful colonisation as a result

of arrival as a spore is highly dependent on whether suitable conditions for germination are present, such as presence of readily assimilable nutrients, lack of competitors and a favourable microclimate. Satisfaction of these conditions results in germination of the spore followed by the formation of a branched network of undifferentiated hyphae. In this early stage of development growth is exponential, however as the colony expands the nutrient conditions within the center soon become different from those at the periphery. The region of hyphae located at the colony margin is termed the marginal or peripheral growth zone. Differentiation of the mycelium in many fungi may start with the formation of septa.

Another important differentiation event in higher fungi is anastomosis or hyphal fusion. Anastomosis may occur when two lateral branching hyphae come in close proximity to each other. The main role of anastomosis is to convert a radial system of hyphae into a three-dimensional network, thus allowing protoplasmic continuity between the whole mycelial colony. Moreover, anastomosis can also have important roles in the recognition of self- / non-self hyphae during mating or combat responses (Rayner & Boddy, 1988; Ainsworth, *et al.,* 1992).

As indicated above, colonisation may also be initiated as mycelium. In contrast with arrival as spores, mycelial colonisation is not as susceptible to unfavourable nutrient conditions due to the mycelium's ability to import nutrients and water from other connected sites of mycelial activity. The mycelium may colonise a resource over a broad front, with the concerted action

of many individual hyphae of the same genotype. Alternatively, highly specialised multihyphal structures such as rhizomorphs and mycelial cords may be the mode of arrival (Rayner & Boddy, 1988).

Mycelial cords are produced by a variety of fungi and in the natural environment they are often found at the interface of surface litter and soil in deciduous woodlands (Boddy, 1993). Cord formation may be induced by a number of stimuli such as reduced water potential, low nutrients, copper, calcium or the presence of antagonistic fungi (Watkinson, 1999). Mycelial cords can exhibit a diversity of forms, ranging from diffuse fans of mycelium which become consolidated a few centimetres behind into aggregations of parallel, longitudinally aligned hyphae to true rhizomorphs (see below). Hyphae within the cord may autolyse leaving wide channels permitting the translocation of water and nutrients to the advancing mycelial front. Several studies have highlighted the importance of mycelial cord formation in the foraging (Dowson, Rayner & Boddy, 1986; 1988) and combat (Coates & Rayner, 1985; Chapela *et al.*, 1988) strategies of basidiomycete fungi.

Rhizomorphs have a similar function to mycelial cords however they consist of a root-like apical 'tip' rather than the advancing mycelial front associated with cords (see Figure 1.1). The most extensively studied rhizomorphs are those of *Armillaria mellae*, the honey fungus. Probably the most important feature of rhizomorphs is that they can extend several times faster than diffuse marginal mycelium (Rayner & Boddy, 1988).

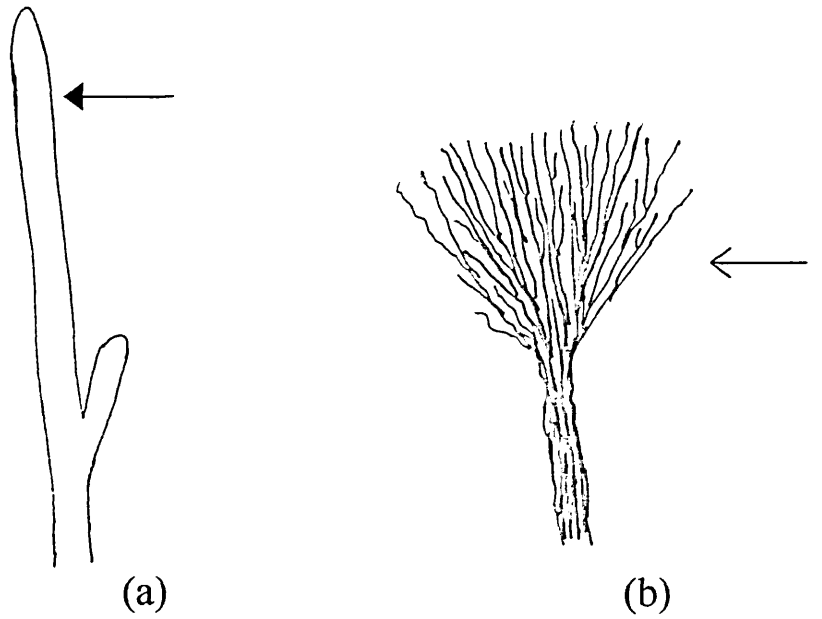


Figure 1.1. An example of two forms of linear hyphal aggregate formed by basidiomycete fungi. (a) Rhizomorphic growth, closed arrow indicates region of apical growth. (b) Mycelial cord growth, open arrow indicates diffuse hyphal front with loss of apical control over extension. Adapted from Rayner and Boddy, (1988). See text for details.

1.2.3.2. Establishment

The second stage of colonisation is establishment. During this phase the fungal thallus must gain access to, and command over the available resource. Two methods of resource capture have been identified based on whether the resource is occupied by a competitor organism. Primary resource capture occurs in a resource that is unoccupied by any competitors and the main objective is to obtain maximal colonisation before potential competitors begin to establish themselves. Certain types of wood decaying fungi display

developmental patterns that increase their efficiency at resource capture. One such phenomenon is the 'slow dense / fast effuse' dimorphism (Coates & Rayner, 1985). In this developmental pattern the mycelium can change the angle and frequency of lateral hyphae branching, thereby occupying more or less resource.

In contrast to primary resource capture, secondary resource capture occurs after the resource is occupied by other organisms and requires active combative mechanisms. Invasion of the resource is usually slow and characterised by the invasion of the resource by a mycelial front. Further discussion of secondary resource capture and its role in combative interactions will be discussed in 1.3.

1.2.3.3. Exploitation & Consolidation

Exploitation describes the processes involved in the breakdown of recalcitrant substances within the resource as nutrients such as simple sugars are exhausted. Again as was indicated with establishment, the mycelium may change morphology in response to prevailing conditions, thus optimising its efficiency. One such response is an increase in branching of hyphae, which in turn also contributes to the consolidation of the fungal thallus. Consolidation is important for the fungus to resist competitors and/or adverse abiotic factors within its resource domain. Certain fungi, especially those causing white rot, produce specialised mycelial structures termed pseudosclerotial plates. These are composed of highly branched and often melanized hyphae which are resistant to both mycelial and fluid penetration.

1.2.3.4. Exit

For continued existence, the fungus must be capable of exiting the resource as nutrients become exhausted or conditions (biotic and/or abiotic) become unfavourable. Exit from a resource may be effected by either formation of reproductive spores or by outgrowth as mycelium. As with arrival, mycelial exit may occur as mycelial cords or rhizomorphs. It is important to recognise that the mode of exit is highly dependent on the ecological strategy of the organism. Ecological strategies will be discussed in more detail in 1.3.1. However, in general, ruderal species tend to exit a resource via production of asexual spores, however stress tollerant and competitors, which tend to be persistent, have a greater capacity for production of biomass may exit as either mycelium or the production of large fruiting bodies (e.g. bracket fungi often found in woodlands) (Cooke & Rayner, 1984).

1.2.4. Mechanisms of mycelial organization

As has been indicated in the forgoing, the fungal mycelium can display considerable diversity in its developmental pattern as it encounters environmental uncertainty (e.g. dimorphisms, formation of reproductive structures, mycelial cords, rhizomorphs etc.). Furthermore, due to its indeterminate nature, it is possible that the mycelium may span a variety of different environments, displaying different behaviour in each. It is therefore worth while considering how the mycelium regulates the variety of functionally distinctive forms it can display. Rayner & Boddy (1988) termed this behavioural change of mycelia as 'Functional modes', defined as *"programmed morphogenetic cycles or events which equip the fungus with the capacity to adapt its behaviour to accord with local environmental pressures"*. It is thought that the basis

of these mechanisms is at the molecular level, via differential gene expression, which is presumably induced by exogenous environmental factors (Sharland, Burton and Rayner, 1986; Rayner and Coates, 1987; Rayner & Boddy, 1988). However, it is unclear whether exogenous factors affecting developmental patterns operate through direct effects on metabolic activity (i.e. a continuum of feedback responses related to quantitative level of stimulus) and/or, by cueing endogenous genetic switching mechanisms resulting in an abrupt change in morphogenesis (Rayner and Boddy, 1988). The inherent difficulties associated in determining how genetic switching mechanisms operate lead to the development of a non-gene expression view of mycelial morphogenesis. This hypothesis considers the patterns produced by the mycelium as an automatic consequence of the way that it is organised as an assimilative and distributive system (Rayner, 1991; Rayner *et al.*,1994). This theory considers the mycelium as an interconnected structure, responding to changing circumstances by genetically encoded mechanisms. Feedback processes regulate metabolic pathways that may lead to both rigidification and sealing (insulation) of external boundaries or to the onset of degradation reactions (Rayner, 1994). The mechanism of mycelial insulation is thought to involve the production of hydrophobic, aromatic and terpenoid compounds, certain polypeptides known as 'hydrophobins' and the operation of phenol oxidising enzymes resulting in melanisation (Wessels, 1991). These processes are also of fundamental importance in the resistance and antagonistic mechanisms involved during fungal interactions.

1.3. THE ECOLOGY OF FUNGI

In the previous section the growth and development of the individual fungal mycelium was outlined. However, in the natural environment fungi tend to exist as dynamic communities consisting of assemblages of organisms which interact with their surrounding abiotic and biotic environment. Understanding the pattern and occurrence of an organism (or population) is essentially a problem of identifying its realised niche. This task involves the identification of, not only the space-time occupied by the organism in nature but also its role within that space-time (Cooke & Rayner, 1984). This in turn leads to the problem of how best to classify the role of an organism. One method suggested by Rayner & Boddy (1988) is the identification of spatial activity domains. In this concept the domain of an organism is characterised by the physical space in which it occupies and by its activity within that space. An interaction can be said to occur when activity domains overlap.

1.3.1. Ecological Strategies

In addition to spatial activity domains, the classification of ecological strategies has also been suggested as useful in understanding the pattern and occurrence of fungi. The ecological strategy of an individual can essentially be defined as the behaviour it displays at any particular stage in its life cycle (Pugh & Boddy, 1988). These strategies are important in understanding the adoption of either an ephemeral or a persistent life form (*r-K* selection). *r-K* selection is determined by environmental stress, incidence of competitors and ecological disturbance; which in-turn dictate the primary ecological strategies exhibited by fungi. These strategies, termed, ruderal, combative, and stress tolerant, may be used to define the behaviour exhibited at a particular time by an organism, but cannot be used to classify an organism *per se,* since changes may occur under different circumstances or at different life-cycle stages (Rayner & Boddy, 1988).

It is also important to recognise that most organisms probably exhibit combinations of the primary strategies during their life cycle. They may be said to display secondary (a combination of two) or tertiary (a combination of three) ecological strategies. The characteristics of the three ecological strategies can be summarised as: 'ruderal', resulting in effective dispersal, early arrival and early exit as stressful or combative conditions intensify; 'combative', allowing effective defence or attack of a resource domain as the potential incidence of competitors increases in the relative absence of stress or disturbance; and 'stress-tolerant', resulting in tolerance or optimisation of stressful conditions which limit the incidence of competitors.

1.3.2. Fungal interactions with the abiotic environment

As briefly discussed in 1.1 the most significant interaction fungi have with their abiotic environment is nutrient acquisition. However, there are many other abiotic factors that influence the growth and behaviour of the fungal mycelium. Fungal growth and activity in artificial culture are influenced by temperature, pH, water availability and gaseous composition (e.g. Low, 2000; White & Boddy, 1992 & White *et al*, 1996). Furthermore, the developmental behaviour of an individual may also change in response to extremes of certain abiotic factors. For example, temperature imposes physiological limits on, and controls the rates of spore germination, reproduction and may also be influential in determining the outcome of competitive inter-specific interactions (Carreiro & Koske, 1992). In the natural environment, the daily and seasonal temperature and water availability may vary considerable, and indeed may induce certain morphogenetic behaviours such as the formation of fruit bodies, sclerotia, or mycelial cords.

1.3.3. Interaction with the biotic environment

In the natural environment all organisms come into contact or close proximity with numerous others, therefore frequent interactions must be an important feature of their pattern of life (Rayner & Boddy, 1988;White & Boddy, 1992). Knowledge of both inter- (between species) and intra- (within species) specific interactions between fungi, and between fungi and other organisms are of vital importance in understanding the structure and functioning of fungal communities.

According to a schema proposed by Rayner and Webber (1984) the various types of interaction have been classified. An illustration of this schema is shown in Figure 1.2. The interaction between two individuals can be primarily termed competitive, neutralistic or mutulistic, depending respectively upon whether the outcome is detrimental to both, detrimental to neither but not beneficial to both, or beneficial to both. Competitive interactions can be further subdivided into two further classes, primary resource capture and combat.

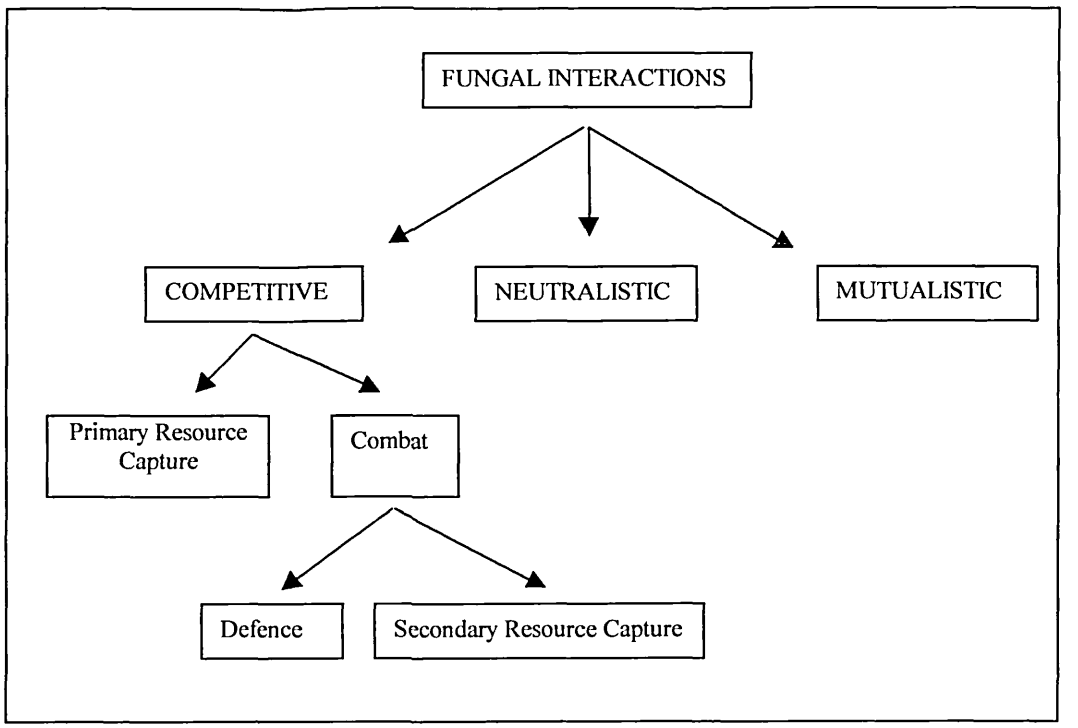


Figure 1.2. Schema used to describe interspecific fungal interactions. Based on Rayner & Webber (1984). See text for further details.

Primary resource capture, as described in 1.2.4.2, is dependent on priority of arrival and sequestration of resources before spatial domain's overlap. Species that are successful at primary resource capture often display features of ruderal ecological strategies, such as effective spore germination, rapid mycelial extension rates and the possession of suitable enzymes to utilise available resources (Rayner and Webber, 1984). Combat interactions occur when the spatial activity domain of two individuals come into proximity and involve active antagonistic mechanisms. These mechanisms may be further classified by whether the domain captured during primary resource capture is defended or invaded by secondary resource capture.

Combative fungal interactions may operate at three distinct levels, hyphal, mycelial and combat at a distance (e.g. antimicrobial substances). Hyphal interactions are thought to involve mechanisms that share common developmental pathways (Rayner, 1986; Rayner and Boddy, 1988). Hyphal interactions can be classed as hyphal fusion (anastomosis), hyphal interference, or parasitism. Hyphal fusion normally results in a rapid rejection response followed by vacuolation and degeneration of the hyphal compartment similar to those seen in somatic incompatibility responses between two dissimilar genotypes. Hyphal interference is defined as when contact but not fusion between two hyphae leads to the death of one or both of the hyphae involved. Parasitism of one hypha by another is normally characterised by either penetration of the host hyphae or by a directional response, such as coiling, after contact is made.

1.4 THE STUDY OF FUNGAL DECOMPOSER COMMUNITIES

1.4.1. In – situ study of fungal communities

An initial stage in the study of fungal communities is the identification and classification of the spatial distribution of organisms and their activity within a particular resource. In general, two different, but complementary approaches have been adopted. These are direct and indirect methods. Direct methods include the identification of sporophores (Vogt *et al.*, 1992), observation of decay and discoloration patterns in wood logs (Rayner & Todd, 1979; Coates & Rayner, 1985), and the use of light and electron microscopy (Wilcox, 1964; Gurr, 1965; Findlay & Levy, 1969). Furthermore, the application of molecular based methods have recently been developed, such as immunological (Goodell & Jellison, 1986; Palfreyman *et al.*

1987; Dewey, 1992) and DNA based techniques (Egger, 1992; Bruns & Gardes, 1993; Gardes & Bruns, 1993). In contrast to direct methods, indirect methods are all dependent on the removal of samples from colonised material and their placement on artificial culture media to allow the outgrowth of resident organisms. A significant problem associated with indirect methods is the selective nature of artificial media. Often this feature has been utilised if particular taxa are sought. For example, Hunt & Cobb (1971) developed a media selective for basidiomycetes, while Russell (1956) developed a media for the selection of white-rot fungi.

The data produced from *in-situ* studies of fungal decomposer communities indicate that their structure and dynamics are highly complex (e.g. Rayner & Coates, 1987; Chapela & Boddy, 1988a, 1988b; Rayner & Boddy, 1988). However, the data produced from such studies is often qualitative, therefore, it is difficult to test theories on the effects of various factors, such as environmental heterogeneity, in an experimentally rigorous fashion due to a lack of suitable experimental models. Therefore, the use of *in-vitro* studies has been useful as a means towards producing more quantitative data of community dynamics.

1.4.2. In–vitro fungal community studies

Most *in-vitro* studies on fungal community dynamics have centred on the interaction between a limited number of individuals under experimentally defined environmental conditions. It is therefore possible to quantify the responses of individuals to specific abiotic and biotic factors. One approach, which has received considerable attention, is the confrontation between two organisms on a common nutrient source (Figure 1.3).

Cooke and Rayner (1984) identified three possible outcomes when two mycelial systems interact in such studies: (1) neutral intermingling of hyphae, (2) deadlock, in which neither mycelium can enter the territory occupied by the other, or (3) replacement, of one mycelium by the other. Interaction events can also occur before mycelial contact due to the diffusion of chemical substances (such as antibiotics) through the growth medium, or even through the air in the case of volatile substances (Bruce, Austin and King, 1984). During the confrontation of two species a diversity of responses may be prevalent. The extension of one or both colonies may be reduced in combination with the production of pigments, massed hyphal fronts, cords, pseudosclerotial plates (specialized defensive organs) or lysis zones. Eventually, one mycelium may grow through the other mycelium resulting in either partial or total replacement.

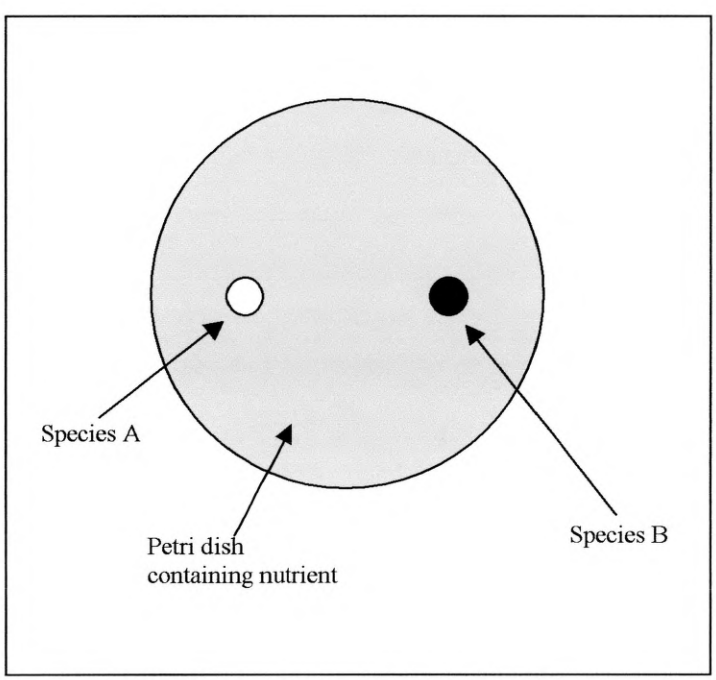


Figure 1.3. Diagram showing pairwise interaction of two mycelial fungal individuals. See text for details.

Data produced from combative interaction studies are often used to rank species in order of their combative ability. According to general ecological terminology combative hierarchies may be either transitive or intransitive (Boddy, 2000). A transitive hierarchy defines a situation where species A is more combative than species B, and both are more combative than species C (i.e. A>B>C). Alternatively, an intransitive hierarchy refers to a situation where species A is more combative than species B, species B is more combative than species C, but species C is more combative than species A (i.e. A>B, B>C, C>A). It can be seen from this that predictions of fungal community development may be extremely difficult, if not impossible, if the species under investigation display an intransitive hierarchy.

Although laboratory based interaction studies in nutrient agar can provide valuable information on the interactive behaviour of fungal species, it must be recognised that extrapolation to the field must be made with caution, as results in artificial culture may not always reflect those in natural substrata (e.g. Dowson, *et al.*, 1988; Griffith & Boddy, 1991; Score, *et al.*, 1998). For example, *Trichoderma harzianum* replaced the dry rot fungus *Serpula lacrymans* in nutrient agar culture under ambient conditions, but deadlocked and was then eventually replaced when paired in wood (Score, *et al.*, 1998). A possible explanation for this difference may be related to the combative mechanism employed by *T. harzianum* (i.e. via the production of antibiotics and volatiles). The action of such a mechanism is likely to be more effective in nutrient media based systems where antibiotics have the potential to diffuse through the media. Agreement between agar based studies and in natural substrata is also likely to be influenced by environmental complexity. Environmental factors have been shown to influence the outcome of interactions in agar culture (Boddy, 2000). Water potential,

gaseous regime and temperature have all been shown to significantly influence the outcome of interactions (Boddy, *et al.*, 1985; Dowson, *et al.*, 1988). Furthermore, the size and quality of the resources have also been shown to influence interaction outcome. For example, fungi that occupy large domains within wood resources have been shown to commonly display a higher success in combat compared to those occupying smaller domains when challenged by the same species (Holmer and Stenlid, 1993). Therefore, in the natural environment where resources and abiotic factors are heterogeneous it is likely that the outcome of interactions will display some degree of stochasticity. Indeed, there is growing awareness of the stochastic nature of the fungal mycelium even in agar culture, where a fungal individual can often display a variety of responses when subjected to ostensibly the same set of conditions (e.g. Halley *et al.*, 1996; and Gwyn S. Griffith, personal communication). Such behaviours are quantitatively most noticeable during the assessment of interaction studies in nutrient agar confrontation studies, even between a few studied replicates.

Although the benefits of *in-vitro* interaction studies in understanding the factors that influence the community dynamics of fungi are clear, a major limitation exists in how such small-scale studies (i.e. one or two species at a time) relate to the behaviour at larger 'community' scales. At larger scales it is probable that contrasting emergent behaviours may arise because individuals in a group or patch behave differently to individuals that are isolated. Processes such as modification of the environment, resource translocation and hyphal networking or anastomosis (e.g. Rayner *et al.*, 1994; Rayner, 1996) may all influence community development. Issues relating to scale have been a central problem in ecology over the last fifty years (Allen, *et al*, 1993). Levin, (1992) emphasised that understanding and predicting the effects of large-scale features

of the global ecosystem, such as global biodiversity, climate change, and the distribution of greenhouse gases and pollutants, all have origins in and consequences for fine scale phenomena. Therefore, in the development of a complete understanding of how the fungal community functions it is necessary to address issues relating to scale. However, although the use of *in-vitro* fungal interaction studies permits the quantification of community dynamics, the effects of complex parameters such as spatio-temporal heterogeneity and scale are often lost.

1.4.3. Modelling approaches of fungal communities

From the foregoing it is clear that significant limitations exist in both field and laboratory based studies of fungal communities. Indeed, a complete understanding of the natural environment based on observation alone is not only involved but also unmanageable due to its complexity. The use of conceptual representations, known as models, has therefore been instrumental in expanding the boundaries of our understanding of the natural environment. A model in its broadest sense is a partial, simplified version of a real life thing (Barrow, 1999). Models may take various forms, ranging from scale models of ships, planes, and railways, to more abstract mathematical models such as Newton's equations that describe the orbit of the planets around the sun (Holland, 2000). The main value of models is that they allow us to first represent and then make measurements and predictions that would be otherwise awkward. For example, during the design of aircraft, engineers often use scaled down models of aeroplanes to test their aerodynamic properties in wind tunnels, as it would be impractical and costly to perform such tests at full scale.

Mathematical models based on experimentally observed characteristics of fungi have

been increasingly used in formulating hypothesis about their interactions (Carlile and Watkinson, 1994). The advantage of such models is interplay between quantitative experimental data and theoretical predictions. It is therefore possible to test the model by predicting the result of, for example, changing an environmental factor in the experimental system and then observing whether the predicted result occurs. Furthermore, appropriate theoretical models should allow inferences to be made regarding the underlying mechanisms or processes involved during fungal interactions.

Most of the models used in mycology have been mainly concerned with attempts to explain the growth and development of hyphae and mycelia in relation to their abiotic environment, and have been extensively reviewed by Ritz & Crawford (1999). Modelling approaches to fungal communities, however, have received less attention. Indeed, the work of Halley *et al* (1996) represents the only attempt to model a multi-species fungal community. In this work, a computer based simulation model known, as a 'cellular automaton' was developed to predict the decomposition of wheat straw by four saprotrophic fungi. The model was based on real experimental data obtained by Robinson *et al.* (1993) during studies on resource capture by interacting fungal colonisers of straw. Although this model was capable of reproducing some of the behaviours exhibited during the experimental study, validation of the model was only possible on a qualitative basis. Furthermore, the model also ignored aspects of the fungal community such as sporulation and the co-ordinated behaviour of the mycelium. The results described in this thesis have also been used in the development of a cellular automaton model (Bown, *et al.*, 1999; Bown, 2000).

1.5. ORIGINS OF THE PROJECT

1.5.1. The Frigate Unicorn

The Frigate Unicorn (Figure 1.4) is the oldest British built ship afloat and is also one of the ten oldest ships afloat in the world. Since her launch in 1824 she has never been commissioned for active service by the Royal Navy, which combined with the construction of a roof over the upper deck, may contribute to her remarkably sound structural condition, comprising of approximately 90 % of her original English Oak timbers.

White *et al.*,(1996) initiated work to characterise the prevailing microclimate and the fungal community structure of the keelson and sister keelsons of the Frigate Unicorn. One of the main objectives of this work was to aid the development of suitable management strategies for the conservation of the ship. In particular, the effect of environmental disturbance on the structure and activity of the decay community was of key importance, due to a proposal to dry-dock the ship.

White *et al.*,(1996) isolated at least 14 different species from the keelson and sister keelson and found that most of the sampled cores were of poor structural integrity (Figure 1.5). Based on the characteristics of the species isolated and the high moisture content of the cores, White *et al.*,(1996) suggested that decay of the keelson was a relatively recent event, most probably due to the ingress of rainwater through

Figure 1.4. Photograph of the Frigate Unicorn at Victoria Dock, Dundee, Scotland.

the then leaking roof of the ship. Moreover, statistical analyses of the community structure suggested that timber moisture relations and the combative hierarchy of species did not solely direct the colonization process and that other factors such as the spatial occurrence and concentration of inoculum were also likely to be of importance in determining community development. These findings suggest that the underlying processes of community development (i.e. the various interactions of the fungal species) are likely to be extremely complex. However, these explanations are extremely difficult to validate *in situ*. An alternate approach could be to develop a model to investigate some of the factors proposed to influence the fungal community development within the keelson of the Frigate Unicorn.

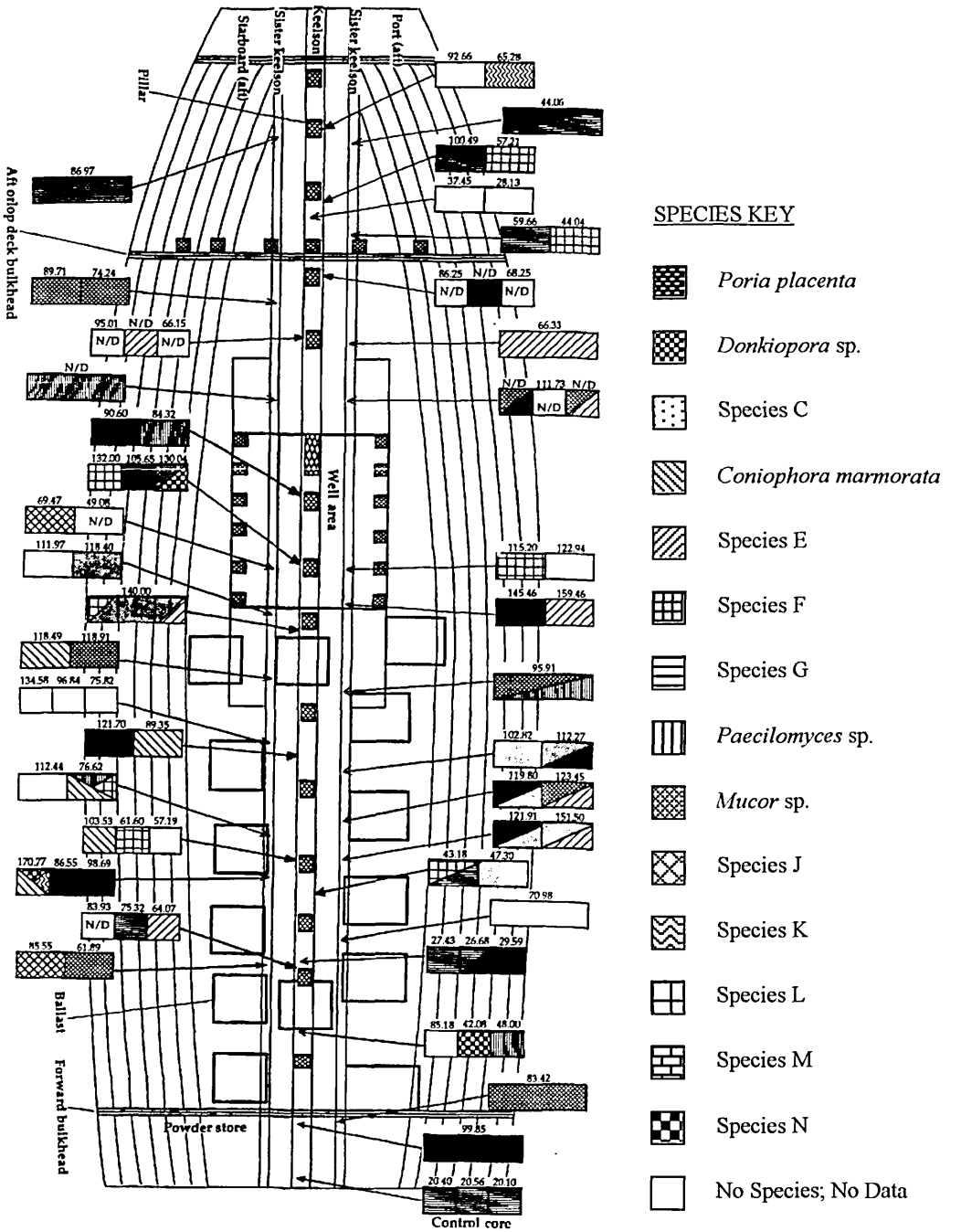


Figure 1.5. A plan of the hold of the Frigate Unicorn. Patterned boxes represent the location within the keelson and sister keelsons, from which cores were sampled, the species isolated, together with the moisture content off the core portions. Reproduced from White *et al.*, (1996) with permission from Dr N. A. White.

1.6. AIMS

The main aim of this thesis was to develop a suitable biological, experimental model, which permits the controlled study of the spatiotemporal dynamics of fungal communities. Specifically, the model will be used to determine whether the behaviour of small-scale, pairwise confrontations can be used to describe the outcome of more complex large-scale, multi-species fungal interactions and investigate the importance of various factors, such as spatial distribution of species and inoculum potential, on the development of multi-species fungal interactions.

An account of each phase of this research is subsequently described;

- Description of the methodologies used in the whole research programme (Chapter 2).
- Determine of the key experimental parameters influencing the outcome of small-scale two species interactions (Chapter 3).
- Determine the effects of scaling and patch size on the interaction dynamics of two species interactions (Chapter 4).
- Determine the effects of scaling and species spatial distribution on large-scale three species interactions (Chapter 5).
- Investigation of the occurrence of a morphological variant in the *P. placenta* isolate used in the interaction studies (Chapter 6).
- Development of a non-destructive method to analyse interaction outcome (Chapter 7).
- Final discussion and conclusions (Chapter 8).

CHAPTER 2: MATERIALS AND METHODS

2.1. SOURCE OF EQUIPMENT AND MATERIALS

Suppliers of equipment and materials are listed in Appendix A.

2.2. FUNGAL ISOLATES

The three fungi used in this study were isolated from the keelson of the Frigate Unicorn by White *et al.*,(1996). The fungi were provisionally identified *as Poria* (*Postia*) *placenta* (= *Tyromyces placenta* or *Oligoporus placenta*) (Fr.) Cooke sensu J. Ericksson, *Coniophora marmorata* Desm. and *Paecilomyces variotii* Bainier, (viz. species A (Unicorn isolate 28b), D (Unicorn isolate C1) and H (Unicorn isolate A1)), respectively. In this thesis, all figures, tables and the classification of interface and state transition classes (see 2.4.1.2), use abbreviated species names for sake of clarity. These were *Pp* for *P. placenta*, *Cm* for *C. marmorata* and *Pv* for *P. variotii*.

Furthermore, during the project the morphology and physiological characteristics of *P. placenta* changed during experiments described in 3.3.5 and 4.2. An investigation of this phenomenon is reported in Chapter 6. *P. placenta* isolates were therefore denoted as *P. placenta* [1], [2] or [3] based on the features, 'obtained from original stock slopes (White *et al.*, 1996)', 'morphologically distinct form of *P. placenta*[1]' or 'wood grown form of *P. placenta*[2]' (see 2.6.3), respectively.

2.3. CULTURE OF ORGANISMS

Cultures of each fungal isolate were inoculated onto 2% (w/v) malt extract agar, (MEA; 20 g oxoid malt extract, 15 g oxoid technical agar in 1 l distilled water (dH_2O), approximate pH 5.6) autoclaved at 121 °C for 20 min, then incubated at 25 °C in the dark until the agar surface was approximately 80% covered with mycelium. Stock slopes of 5% (w/v) MEA were prepared and inoculated with 7 mm cores of mycelium taken from the periphery of agar cultures. Stock slopes were stored at 4 °C in the dark and subcultured every 6 months.

2.4. TESSELLATED AGAR TILE INTERACTION (TATI) SYSTEM

2.4.1. Microcosm design

The basic microcosm design used in all interaction studies was based on that described by Ritz (1995). Square tiles (10 x 10 x 3 mm) of 2% malt extract agar were arranged in either 2 x 1, 3 x 3 or 6 x 6 arrays in Petri dishes (Sterilin). The tiles were separated by 2 mm air gaps unless stated otherwise (restricting diffusion of metabolites, but allowing diffusion of volatiles and formation of bridging hyphae between tiles). The tiles were obtained using a cutting device consisting of a bank of 5 single edged razor blades, separated at 10 mm intervals using spacer pillars (RS Components Limited), mounted on threaded brass rods (RS Components Limited). Two series of cuts (Fig. 2.1a) were made in an agar plate (the second cut was perpendicular to the first (Fig. 2.1b)) thus producing 16 identically sized agar tiles. These tiles were then arranged in the desired arrangement using a sterile microspatula (Fig. 2.1c).

Confrontations were established as either precolonised or simultaneously inoculated tiles. Precolonised tiles were cut from the actively growing margins of 2% MEA cultures and confronted in a radial direction with respect to mycelial growth (Fig 2.2). Simultaneously inoculated tiles were prepared by placing 4 mm diameter cores obtained from actively growing margins (Fig. 2.1d) of 2% MEA cultures, at the centre of individual tiles (Fig. 2.1e). Petri dishes were sealed with strips of Parafilm, placed in loosely sealed plastic bags containing a strip of water saturated tissue paper (to maintain humidity), and incubated at either ambient room temperature (17 - 25 °C) or 15 °C, in the dark. Aseptic technique was maintained throughout.

2.4.2. Assessment of interaction outcomes and data analysis

Microcosm replicates were harvested at various time intervals depending on individual experiments. The spatial distribution of fungal species extant within tiles at a given time point, hereafter referred as the interaction outcome, was assessed by cutting each tile into quarters, then placing each quarter onto fresh 2% MEA plates (Fig 2.3). The plates were incubated for 5 - 6 days and the resulting mycelial outgrowth was identified by visual assessment.

2.4.2.1. Binary tile (2 x 1) tessellations

For all 2 x 1 tessellations, the presence of individual fungal species in each tile was expressed as the proportion of each species existing within each tile quarter. Stepwise logistic regression (Weisberg, 1985) was used to determine if the presence of a particular species was related to inoculation mode (precolonised or simultaneously inoculated), sampling time, species pairing or identity of the subject tile (i.e. the identity of the tile under consideration).

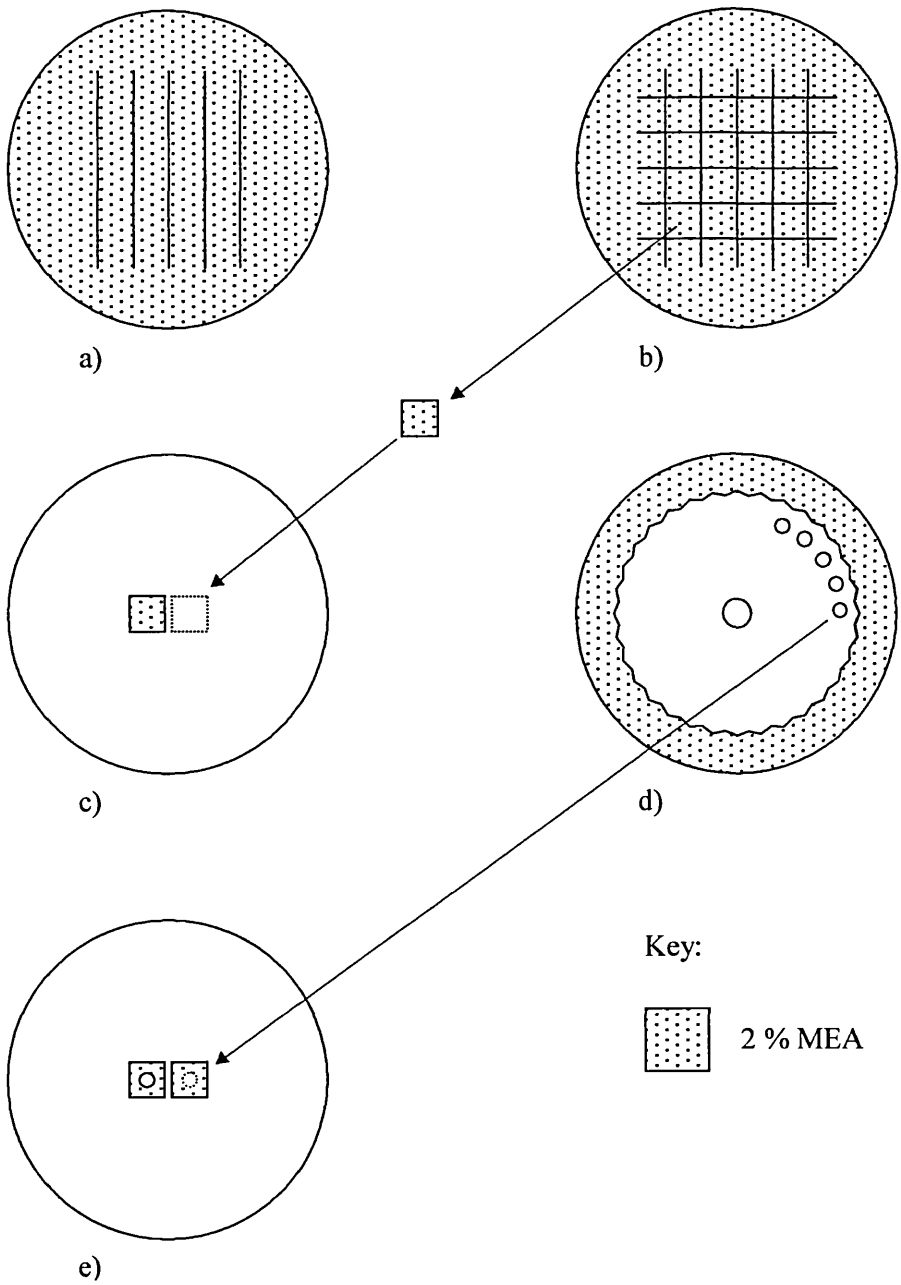


Figure 2.1. Construction of simultaneously inoculated tessellated agar tile microcosm. See text for details. (Scale 1:2)

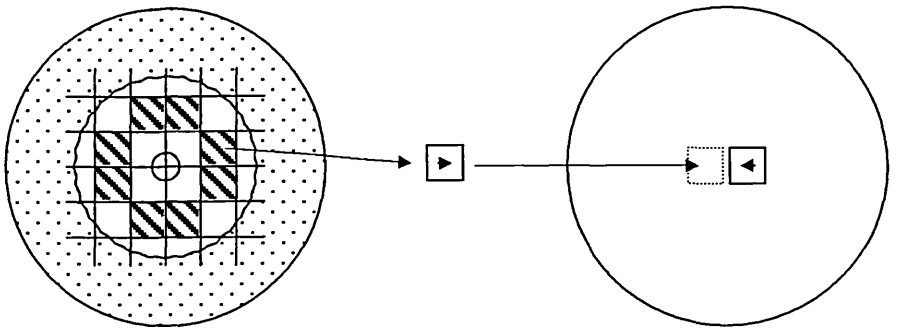


Figure 2.2. Construction of precolonised tessellated tile microcosm. Tiles showing alignment with respect to mycelial growth (arrow head). (Scale = 1:2). See text for details.

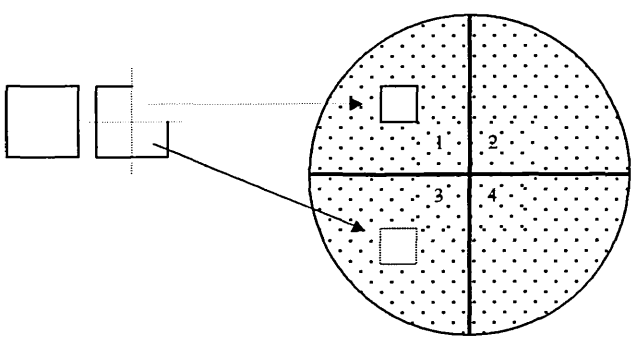


Figure 2.3. Method used to assess interaction outcome of tessellated agar tile microcosms. See text for details. (Scale = 1:2).

2.4.2.2. 3 x 3 and 6 x 6 tessellations

For all 3 x 3 and 6 x 6 tessellations, the presence of individual fungal species in each tile was expressed as the proportion of each species existing within each tile quarter. Data analysis of the large-scale 3 x 3 and 6 x 6 tessellations was carried out using parameters representing the key spatial and temporal descriptors of each tessellation. These were; (i) 'Interface classes', i.e. the total number of interfaces between adjacent tiles of the combinations; *Pp:Pp, Cm:Cm, Pv:Pv, Pp:Cm, Pp:Pv, Cm:Pv.* Where there was multiple-occupancy of tiles, the numbers were expressed as a proportion of the whole tile, i.e. the total number of interfaces scored was constant at 12 for the 3 x 3 and 60 for 6 x 6 tessellations. (ii) 'State transition classes', i.e. the total number of tiles which showed a particular transition from one occupational state at a particular time point, to another at the time of sampling (e.g. *Pp>Cm, PpCm>PpCmPv,* etc.). There were 21 of such classes. Thus for each tessellation, a multinomial data set of 27 values (i.e. 6 interface and 21 state transition) was inputted into a principle component analysis (PCA) using the sums of squares and products (SSP) method within GENSTAT (Genstat 5 Committee, 1993), in order to test for the degree of similarity between the various states of the tessellations.

2.5. ASSESSMENT OF COLONY GROWTH AND CULTURAL CHARACTERISTICS

All measurements of colony growth were made from 9 cm triple-vented Petri dishes containing 20 ml 2% (w/v) MEA, inoculated at their centres with 6 mm cores of respective fungal species. Plates were sealed with strips of Parafilm, placed in loosely sealed plastic

bags containing a strip of water saturated tissue paper (to maintain humidity), and incubated at 15 °C, in the dark. Mean radial extension rates were determined by recording two perpendicular diameters of each colony at daily time intervals. Measurements were performed in triplicate for each species.

The terminologies of Stalpers (1978) and Rayner & Boddy (1988) were used to describe the macro- and micro- morphological cultural characteristics.

2.6. ASSESSMENT OF MORPHOGENESIS IN *P. PLACENTA*

2.6.1. Extracellular enzyme production

Biochemical tests for laccase (EC 1.10.3.2 (Barman, 1969)), tyrosinase (EC 1.10.3.1 (Barman, 1969)), and peroxidase (EC 1.11.1.7 (Barman, 1969)) production were performed for each *P. placenta* culture (*P. placenta*[1] & *P. placenta*[2]) in triplicate according to Stalpers (1978). Plates were flooded with the appropriate test solutions, the excess discarded, then incubated under suitable conditions, and the presence or absence of colour development was recorded.

(i) The laccase-α-naphthol test employed 1.44 % (w/v) α-naphthol (Sigma) in 96% ethanol (Sigma). A purple colouration resulting within 2h indicated the production of extracellular laccase, suggesting the ability to oxidise p-diphenols (Metzler, 1977; Stalpers, 1978; White & Boddy, 1992).

(ii) The tyrosinase test employed 1.08% (w/v) *p*-cresol (Sigma) in 96% ethanol. An

orange - brown colouration developing within 12h indicated tyrosinase activity, suggesting the ability to hydrolyse monophenols and oxidises o-diphenols (Long & Alben, 1978; White & Boddy, 1992).

(iii) The peroxidase test employed equal parts of 0.4% (v/v) hydrogen peroxide (Sigma) and 1% pyrogallol (Sigma) in water. A yellow - brown colouration developing within 2h indicated peroxidase activity (Stalpers, 1978, White & Boddy, 1992).

2.6.2. Isolation of single cultures of P. placenta from hyphal tips

Pure cultures of both *P. placenta*[1] and *P. placenta*[2] were produced via excision of hyphal tips using the following procedure (Carlile & Watkinson, 1994; J. Duncan, personal communication). Five replicates of *P. placenta*[1] and *P. placenta*[2] were inoculated onto Petri dishes containing 10 ml of tap water agar (TWA: 15g Oxoid technical agar in 1 l tap water) overlaid with a sheet of cellophane membrane and incubated at 15 °C for 5 days. Hyphal tips were then excised on small pieces of membrane using a sterile scalpel and transferred to fresh 2% MEA plates. Plates were incubated at 15 °C for 2 weeks and the resulting colonies were examined based on their cultural characteristics (see section 2.5.).

2.6.3. Growth in wood

Pure cultures of *P. placenta*[2] (obtained from experiments described in section 2.6.2) were used to inoculate 3 x 750 ml Nalgene jars containing 200 ml of 2% MEA. The jars were incubated in the dark at 15 °C for 2 weeks. Wood blocks (10 x 10 x 10 mm) of Scots Pine (*Pinus sylvestris*) sap wood were cut then autoclaved twice at 121 °C for 30 mins with a 24 h incubation period between each session. Five blocks were added

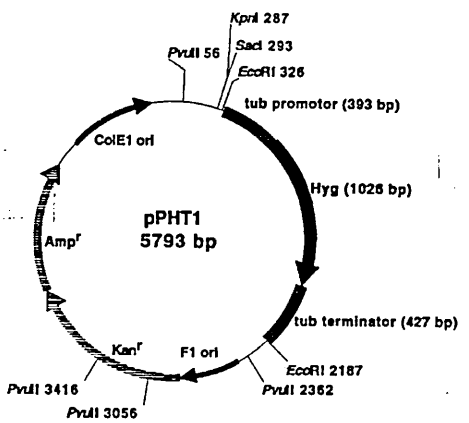
to each pre-inoculated jar, then incubated for a further 2 months after which time they were removed and used to inoculate fresh 2% MEA plates. The resulting species were termed *P. placenta*[3]. These cultures were then incubated at 25 °C and used as inoculum in an interaction experiment to compare the combative ability of *P. placenta*[1], *P. placenta*[2] and *P. placenta*[3] against *C. marmorata* in binary tile tessellations (described in section 2.4.).

2.7. GENETIC TRANSFORMATION OF *C. MARMORATA*

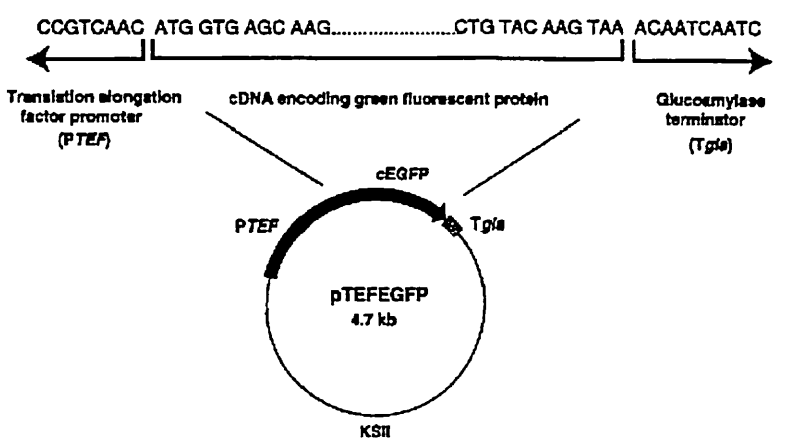
2.7.1. Plasmid DNA preparation

Plasmid pTEFEGFP (Figure 2.4a) (kindly provided by Dr D Cullen, USDA Forest Products Laboratory and Plant Pathology Department, University of Wisconsin, USA), contains a mutant red shifted Green Fluorescent Protein (GFP) cDNA fused to the promoter of the *Aureobasidium pullulans* translation elongation factor (TEF) gene and terminator of the *Aspergillus awamori* glucoamylase (gla) gene (Vanden Wymelenberg *et al*, 1997). Plasmid pPHT1 (Figure 2.4b) (kindly provided by Dr M Zolan, Indiana University, Bloomington, USA), contains the *Esherichia coli* hygromycin B phosphotransferase (hyh) gene fused to the promoter and terminator regions of the *Coprinus cinereus* beta-tubulin (tub) gene (M. Zolan, personal communication). Plasmids were propagated in *E. coli* (XL2 - Blue - MRF, CLONTECH Laboratories, USA) cultured in Luria Broth (LB) (1 % Oxoid tryptone, 1 % NaCl, and 0.5 % Oxoid yeast extract in dH_2O; pH 7, containing 100 (g/ml ampicillin (Sigma)) overnight with shaking at 37 °C. Cells were harvested by centrifugation at 2500 g for 5 mins. Plasmid DNA was prepared using a QIAprep® mini prep kit following the manufacture's

instructions (Qiagen Ltd, UK). Plasmid DNA yield was quantified on a 1 % agarose Tris-borate disodium ethylene diamine tetra acetate (EDTA) (Sigma) (45mM Tris-borate; 10mM EDTA) (TBE) electrophoresis gel, then stored at -20 °C in Tris-HCl - EDTA (TE) buffer (10mM Tris-HCl, 10mM EDTA, pH 8). Linearized Plasmid DNA was prepared by digesting 5 µl of each plasmid with *Eco*R1 (Promega).



(a)



(b)

Figure 2.4. Expression vectors (a) pPHT1 and (b) pTEFEGFP. See text for details.

2.7.2. Protoplast production

Fungal protoplasts are produced through the enzymatic removal of the cell wall in the presence of an osmotic stabiliser. The following methods were used to assess the ability to produce protoplasts from the basidiomycete *C. marmorata.*

2.7.2.1. Protoplast production: method 1

Small cores (5mm) cut from the actively growing margin of *C. marmorata* cultures were used to inoculate 20 ml of a medium consisting of 0.1 % oxoid yeast extract, 1 % glucose (Sigma), and 1 % oxoid malt extract (YMG) in sterile dH_2O. The cultures were incubated in stationary culture for 7 days at 25 °C; then each mycelial mat was aseptically removed, washed twice in sterile distilled H_2O, macerated using a sterile spatula, and filtered by suction onto Watman filter paper No.7 (Merck). The resulting mycelial mass was washed once with distilled H_2O, twice with osmotic buffer (various buffer were assessed, see 7.2.1 for details), and then finally resuspended in 4 ml of osmotic buffer, containing 0.4 % lyzing enzymes (Sigma, Lot Number L967) before incubation for 60 - 90 min at 25 °C. Aliquots (0.1 ml) were removed periodically to observe protoplast formation. After enzyme treatment, 40 ml of osmotic buffer was added to dilute the enzyme. The suspension was then filtered through a coarse sintered-glass filter with suction to separate the protoplasts from the mycelial debris. The protoplast yield was estimated using a haemocytometer before a final wash with 1 M sorbitol (Sigma); 10mM Tris-HCl (Sigma); 25 mM calcium chloride ($CaCl_2$) (Sigma), pH 7.5, (STC buffer) containing 1 % glucose. Regeneration of protoplasts was assessed by performing a serial dilution of protoplasts, plated on YMG Regeneration Agar (1.5 % technical agar, YMG containing 0.5 M sucrose

(Sigma) in 1l dH_2O; pH 5.6), incubated for 5 - 7 days at 25 C, and then counting the number of mycelial colonies formed.

2.7.2.2. Protoplast production: method 2

Plate cultures of *C. marmorata* were grown in YMG liquid medium for 7 days at 25 °C in the dark. Four colonies were aseptically removed, then homogenised using a Waring blender. The mycelia were resuspended in 20 ml of YMG then 5 ml samples were used to inoculate 4 x 250 ml conical flasks each containing 45 ml of YMG. The cultures were incubated at 25 °C in the dark on a shaking platform (100 rpm). The mycelia from 2 flasks were harvested after 24 and 48 h and assessed for protoplast production. Cultures were washed twice in sterile distilled H_20, then twice in 0.5 M mannitol (Sigma) in 50 mM maleic acid - NaOH buffer (pH 5.5), before finally resuspending in 7 ml of a solution containing 0.4 % lyzing enzymes in mannitol maleic-NaOH buffer. The solution was incubated at 25 °C, removing 1 ml samples at 15 min intervals to assess the effect of enzyme exposure on protoplast release and regeneration.

2.7.3. Protoplast regeneration and antibiotic sensitivity

Regeneration of *C. marmorata* protoplasts was assessed by plating 1 ml aliquots of a solution containing 100 protoplasts per ml, on YMG regeneration agar. Plates were incubated for 5 - 7 days at 25 °C and the number of mycelial colonies formed were recorded. The percentage regeneration was assessed for 5 replicate plates. Protoplasts produced using the method described in section 2.7.2.2 were plated onto a selective regeneration agar containing a range of hygromycin B (Sigma) concentrations (0, 25, 50, 75 and 100 $\mu g\ ml^{-1}$). Five replicate plates were prepared, incubated at 25 °C for 5 -

7 days, and then assessed for mycelial growth (0 = no growth, 3 = most growth).

2.8. FUNGAL TRANSFORMATION

The following methods describe the procedure used to introduce the GFP gene and a hygromycin gene into the genome of *C. marmorata*. These studies form part of the development of a non-destructive analysis system to map fungal growth within the tessellated tile interaction system. A detailed description of genetic transformation in fungi is presented in section 7.4.

2.8.1. Fungal transformation: Method 1

Transformations were carried out using the following plasmids; (i) pPHT1 only, (ii) a co-transformation with pPHT1and pTEFEGFP (circular), (iii) pPHT1 and pTEFEGFP (linear), and (iv) a control with no DNA added.

Plasmid DNA (2 μg in 20 μl of TE buffer), 1 - 2 x 10^7 protoplasts in 100 μl of STC, and 25 μl of PEG solution (25 % polyethelene glycol (PEG) 3500 (Sigma), 25 mM $CaCl_2$, 10 mM Tris HCl, pH 7.5) were gently mixed, and incubated on ice for 20 min. An additional 1ml of PEG solution was then added and incubated for a further 5 min at room temperature. The samples were diluted with 2 ml STC and 0.5 ml aliquots were then inoculated onto regeneration agar containing hygromycin B. A range of hygromycin concentrations were assessed (0, 25, 50, 75 and 100 μg ml^{-1}). Plates were incubated at 25 °C for 5 - 7 days before overlaying the plates with 5 ml regeneration agar with a higher concentration of hygromycin (150 μg ml^{-1}). The resulting mycelial

colonies growing through this second agar layer were isolated after approximately 2 weeks.

2.8.2. Fungal transformation: Method 2

pPHT1 DNA (2.5 or 5 μg in 60 μl of TE plus 40mM CaCl2, pH 8) was added to approximately 1 - 2 x 10^7 protoplasts in 100 μl of 1 M sorbitol containing 40 mM $CaCl_2$, mixed gently and incubated on ice for 10 min. Control protoplasts (no DNA added) were treated with 60 μl of TE buffer containing 25 mM $CaCl_2$. Samples were then underlaid with 160 μl of a 44 % polyethylene glycol 3500 in 10mM morpholineethanesulfonoic acid (MES) (Sigma) (pH 6.75) solution, incubated on ice for 10 min, mixed gently, and then incubated for a further 10 min. Samples were then diluted with 1.28 ml of STC and 0.3 ml aliquots were added to 4 ml of 0.7 % agar containing 0.6 M sucrose and YMG which were then poured onto the surface of selective regeneration agar plates (final hygromycin concentration of plates were 0, 25, 50, 75, and 100 μg ml^{-1}). Plates were incubated for 5 - 7 days at 25 °C then assessed for colony formation as described in 2.7.3.

CHAPTER 3. DEVELOPMENT OF A REPRODUCIBLE TWO SPECIES FUNGAL INTERACTION MODEL

3.1. INTRODUCTION

In this chapter the development of a biological model to study the dynamics of fungal communities is described. Two species, inter-specific, fungal interactions were carried out using a novel tessellated agar tile system to determine the relevance and importance of selected experimental parameters. Preliminary studies investigated the effect of inoculation method, sampling time, incubation temperature, and consistency of interaction outcome between binary confrontations of three fungi isolated from a local historic ship, the Frigate Unicorn. Additionally, the outcome of 'calibration' binary tile experiments for the large-scale interactions described in subsequent chapters are reported.

The main objectives of the work reported in this chapter were;

- To develop a novel tessellated agar tile system for the study of inter-specific interactions between three fungal species isolated from the Frigate Unicorn by White *et al.* (1996).
- To determine the interaction outcomes of the three fungi studied during small-scale confrontations.
- To determine the effects of key experimental variables on the interaction outcomes between the fungi studied.

3.2. MATERIALS AND METHODS

The basic design and construction of all agar tile microcosms and the method interaction outcome assessment are described in Chapter 2.

3.2.1. Preliminary two species confrontations

Simultaneously inoculated and pre-colonised 2 x 1 (binary) tile interactions were established in all permutations between fungal species *P. placenta*[1], *C. marmorata, and P. variotii*. Eighteen replicates were prepared and incubated in the dark at ambient room temperature (17 - 25 °C). Six replicates of each confrontation were harvested and their interaction outcomes assessed after 2, 4, and 6 weeks.

3.2.2. Extended time scale two species confrontations

Simultaneously inoculated 2 x 1 (binary) tile interactions were established as in 3.2.1. Sixty replicates for each confrontation were prepared with ten replicates harvested and their interaction outcomes assessed after 1, 2, 3, 5, 11 and 19 weeks. All replicates were incubated at ambient room temperature (17 - 25 °C) in the dark. This experiment was performed 9 months after the experiment described in 3.2.1.

3.2.3. Short time scale two species confrontations

Due to the rapid replacement of *P. variotii* by *C. marmorata* an experiment was designed to harvest the tiles over a period of 8 days. The incubation temperature was lowered in an attempt to slow the rate of the interaction. Confrontations were established between *C. marmorata* and *P. variotii*, as simultaneously inoculated tiles incubated at 15 °C in the dark. Ten replicates of each tessellation were harvested and

their interaction outcomes assessed after 4, 6, 7, and 8 days. In addition, a control experiment was designed to investigate whether *P. variotii* inhibited the growth rate of *C. marmorata* during the confrontation. An uninoculated agar tile was paired against a tile inoculated with *C. marmorata*, and the subsequent growth of *C. marmorata* was mapped across the 'blank' tile. Comparison between the control and the two species confrontations was assessed to determine if the presence of *P. variotii* effected the growth of *C. marmorata.*

3.2.4. Incubation temperature study

To investigate the effect of incubation temperature on interaction outcome, simultaneously inoculated tiles of *P. variotii* and *C. marmorata* were confronted and incubated under a range of temperature regimes. These species were selected for this experiment because the interaction temporal dynamics were significantly shorter compared to that of other species confrontations. Six replicates for each temperature regime were established and incubated in the dark at 20, 25, and 28 °C. Replicates were harvested and their interaction outcomes assessed after 5 days. This experiment was carried out approximately two years after the preliminary binary tile interactions.

3.2.5. Binary tile interactions for two species large-scale (6 x 6 grid) interactions (see 4.2)

Binary tile interactions were established as in described in 3.2.2. Six replicates of each confrontation were incubated at ambient temperature (17 - 25°C) in the dark. Replicates were harvested and their interaction outcomes assessed after 6 weeks. It should be noted that during this experiment the morphologically distinct form of *P.*

placenta[1], *'P.placenta*[2]', was encountered, hence results refer to this species rather than *P. placenta*[1].

3.2.6. Binary tile interactions for 1:1:1 three species experiment

Binary tile interactions were established as in described in 3.2.2. Thirty replicates of each confrontation were incubated at 15 °C in the dark. 10 replicates were harvested and their interaction outcomes assessed after 1, 3, and 5 weeks. This experiment was performed approximately 2 years after the preliminary binary tile experiment described in 3.2.1.

3.3. RESULTS

3.3.1. (a) Qualitative macro-morphological description of P. placenta[1], *C. marmorata and P. variotii interactions for simultaneously inoculated and pre-colonised tiles*

Macro-morphological growth characteristics of each species were consistent irrespective of species pairing. *P. placenta*[1] grew white cottony aerial mycelium, *C. marmorata* grew white cottony / woolly aerial mycelium, and *P. variotii* grew as grey-white downy aerial mycelium that produced orange-brown spores as the mycelium aged giving the colony a farinaceous (powdery) appearance.

A photographic representation of the simultaneously-inoculated tile interactions are presented in Figure 3.1. In general, the interaction between simultaneously inoculated tiles of *P. placenta*[1] and *C. marmorata* showed full tile growth after approximately 5 -

6 days. The production of a yellow interaction line was observed at the leading edge of *P. placenta*[1]*'s* tile after 7 - 8 days where *C. marmorata* hyphae had bridged the air gap between the tiles (Figure 3.1a). Subsequently, the interaction line became dark brown over a period of 2 - 3 weeks. During this time *P. placenta*[1] formed a mycelial flush that progressed over the *C. marmorata* tile. A resulting brown 'reverse' (Stalpers, 1978) pigmentation was observed within both species' tiles, and a yellowing of *C. marmorata* mycelium formed within 4 - 5 weeks (Figure 3.1b).

During the interaction between simultaneously inoculated tiles of *P. placenta*[1] and *P. variotii*, both species colonised their respective tiles after approximately 5 - 6 days, subsequently forming a deadlock interaction (Figure 3.1c & d). It was also observed that *P. variotii* colonies were occasionally formed on uncolonised regions of *P. placenta*[1] tiles, probably due to spread of spores, with subsequent deadlock.

During simultaneously inoculated *C. marmorata* and *P. variotii* interactions, both species colonised their respective tiles after 5 - 6 days. *C. marmorata* subsequently produced a dense mycelial flush over the *P. variotii* tile, completely engulfing it within 2 weeks (Figure 3.1e). However, evidence of sporulation by *P. variotii* remained visible within the *C. marmorata* mycelium for the entire duration of the experiment (Figure 3.1f). In all interactions negligible reduction in colony extension rate was observed for all species during the crossing of the air gap between tiles.

In general, visual analysis of the pre-colonised tile interactions were similar to the respective simultaneously inoculated tile observations with only minor differences in colony growth and tile pigmentation.

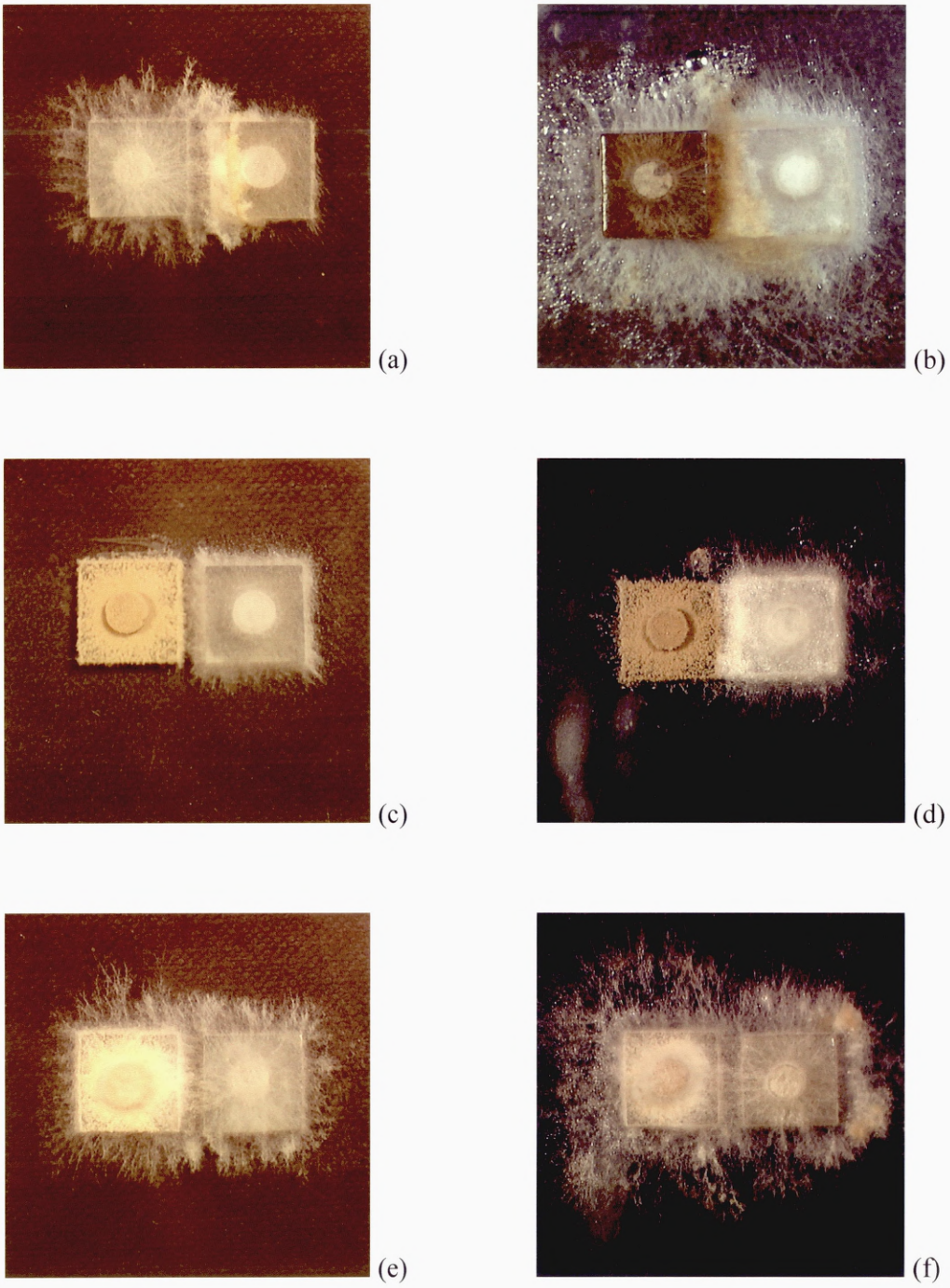


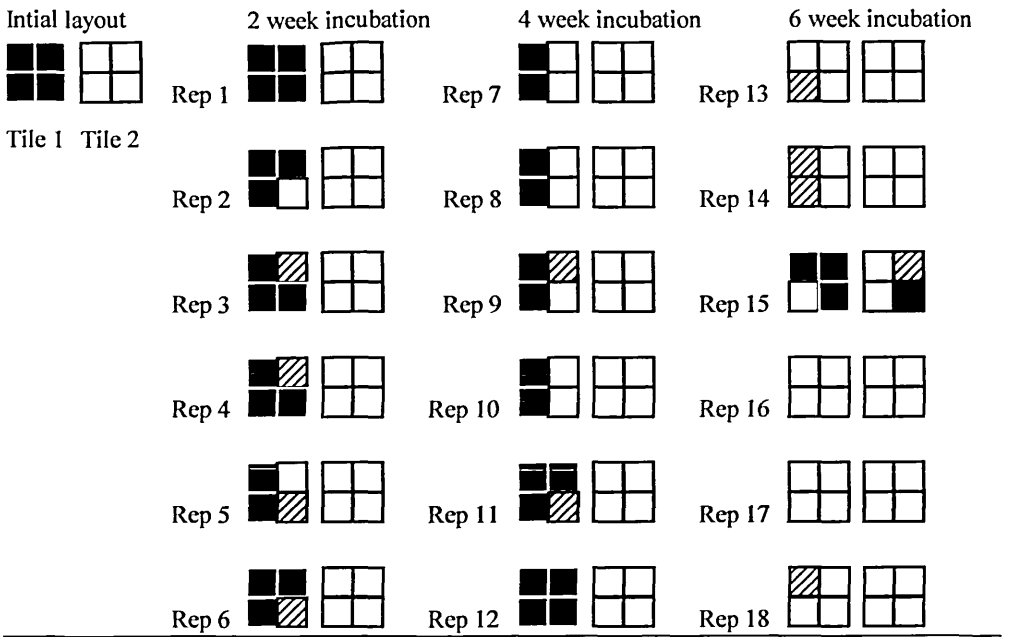
Figure 3.1. Photographs of 2x1 simultaneously-inoculated tile interactions after 2 (a, c, & e) and 5 weeks incubation (b, d, & f). (a & b) *C. marmorata* (left) and *P. placenta* (right), (c & d) *P. variotii* (left) and *P. placenta* (right), (e & f) . *P. variotii* (left) and *C. marmorata* (right). See text for further details.

3.3.1. (b) The proportion of interaction outcomes for 2 x 1 tessellations

The outcomes of the binary tile interactions were expressed as both species distribution maps and as the proportion of fungal species extant within tessellations over time. These data are shown in Figure 3.2 and Table 3.1, respectively. Results indicated that during simultaneously inoculated tessellations; *P. placenta*^{1} usually defended its territory against *C. marmorata*, whilst invading *C. marmorata's* domains; *P. placenta*^{1} usually defended its territory against *P. variotii* and *vice versa*; and *P. variotii* was always invaded by *C. marmorata* whilst *C. marmorata* defended its territory against *P. variotii.*

On more detailed analysis of the results it was apparent that there were differences in outcome between the two inoculation methods. Firstly, after 6 weeks incubation, the pre-colonised tile *P. placenta*^{1} *versus C. marmorata* interaction showed that in 3 out of the 6 replicates *C. marmorata* resisted invasion by *P. placenta*^{1} into it's resource domain compared to the replacement of *C. marmorata* by *P. placenta*^{1} in the simultaneously inoculated interaction. Secondly, there was a higher incidence of co-existence between *P. variotii* and *C. marmorata* in the simultaneously inoculated tiles at week 2 and 4 sampling times compared to the higher occurrence of replacement of *P. variotii* by *C. marmorata* in the pre-colonised tile interactions. Although, after 6 weeks, the interaction outcomes between the two inoculation methods were found to be similar (i.e. *C. marmorata* replaced *P. variotii*). During the *P. placenta versus P. variotii* interaction similar outcomes were displayed for both inoculation methods.

(a) Simultaneously inoculated tiles of *C. marmorata (tile 1) versus P. placenta*[1] *(tile 2).*



(b) Precolonised tiles of *C. marmorata (tile 1) versus P. placenta*[1] *(tile2).*

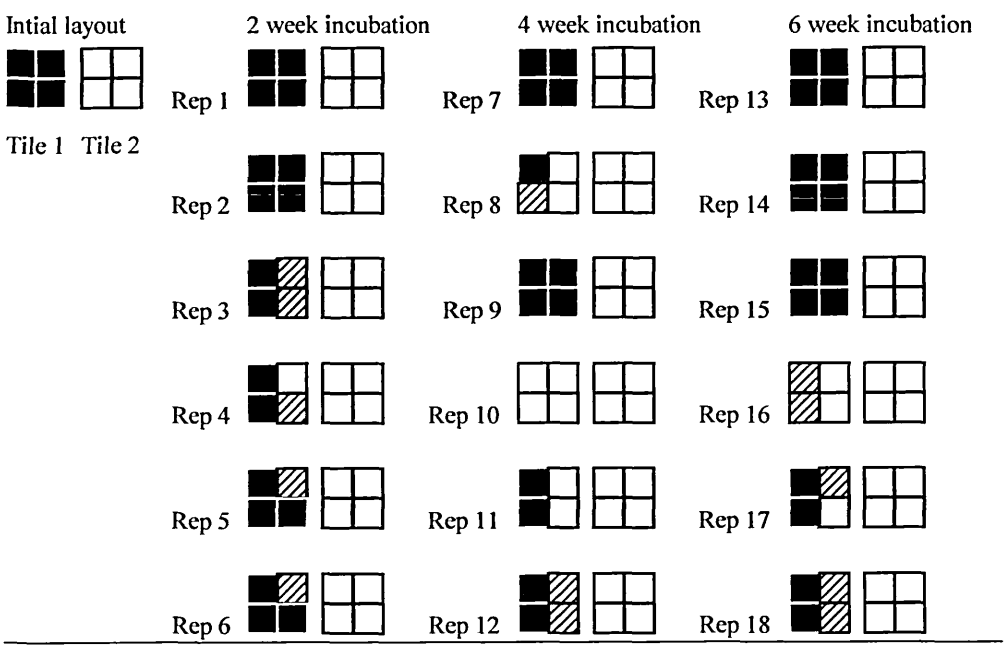
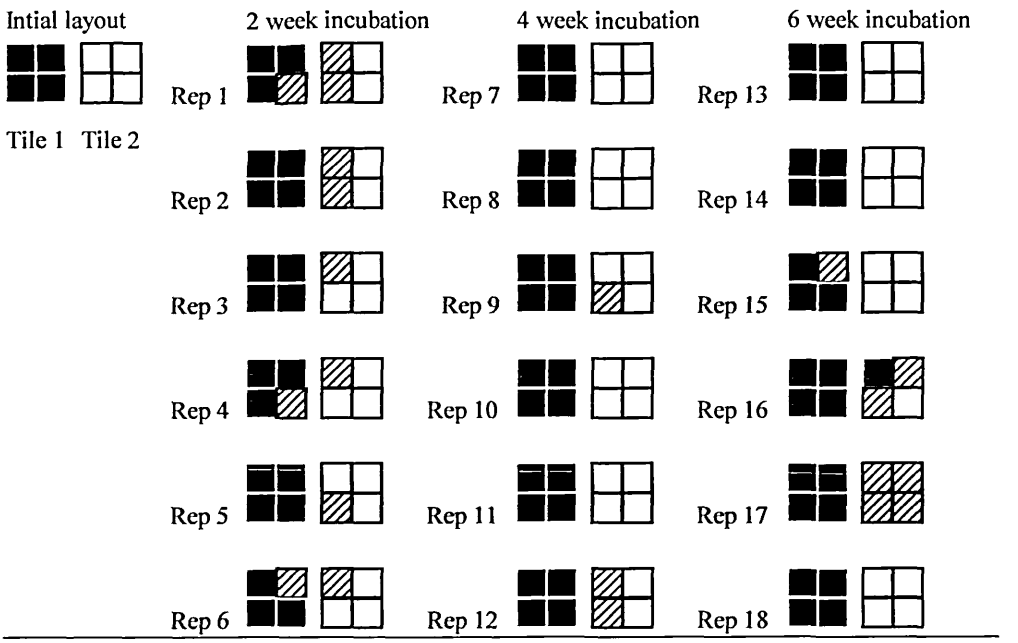


Figure 3.2. (a & b) Species distribution maps of 2 x 1 (binary) preliminary tile interactions. Cross hatched tiles represent occupancy of both species. See text for details.

(c) Simultaneously inoculated tiles of *P. variotii (tile 1) versus P. placenta[1] (tile2*



(d) Precolonised tiles of *P. variotii (tile1) versus P. placenta[1] (tile2).*

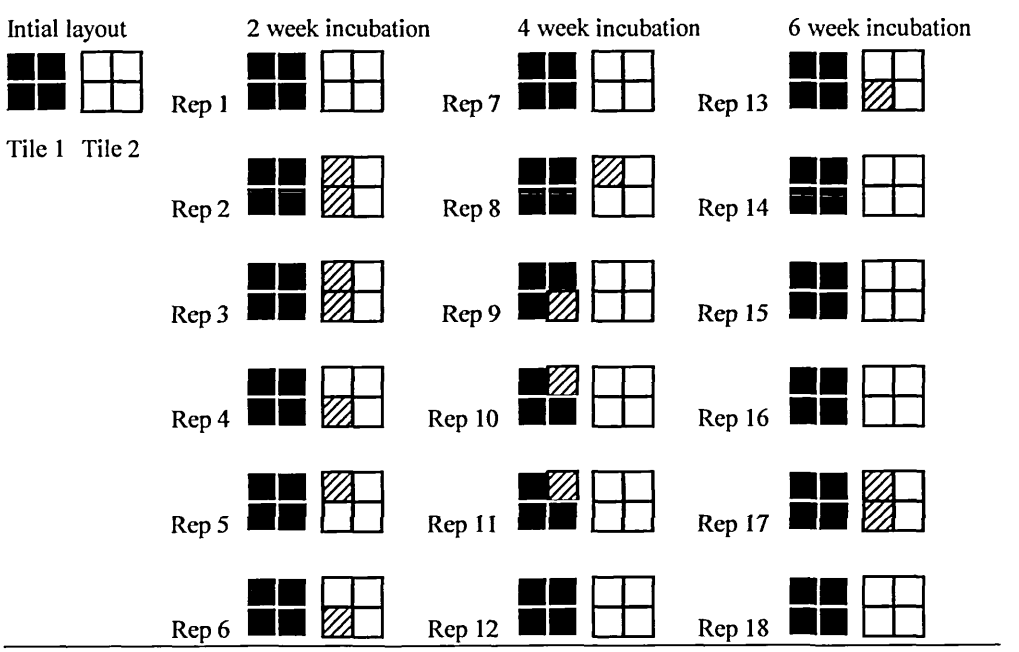
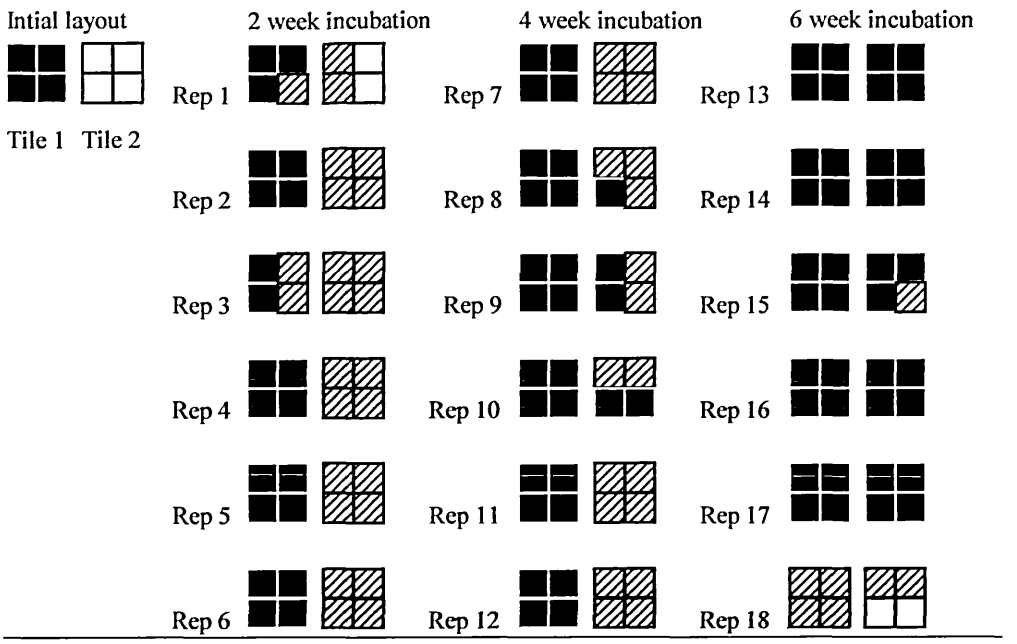


Figure 3.2. (c&d) Species distribution maps of 2 x 1 (binary) preliminary tile interactions.

Cross hatched tiles represent occupancy of both species. See text for details.

(e) Simultaneously inoculated tiles of *C. marmorata (tile1) versus P. variotii (tile2).*



(f) Precolonised tiles of *C. marmorata (tile1) versus P. variotii (tile2).*

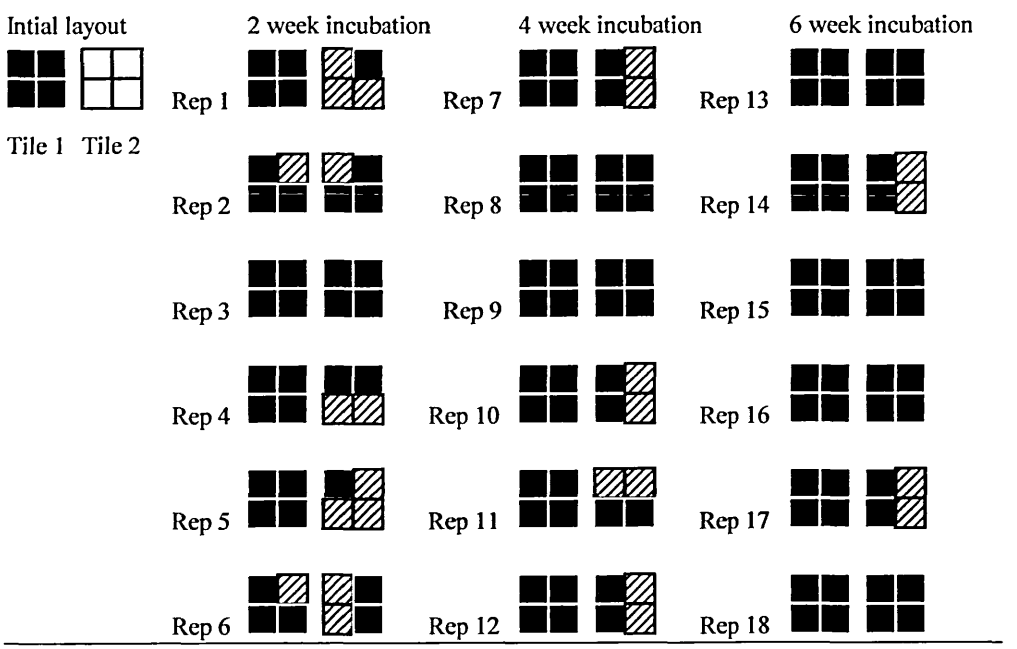


Figure 3.2. (e & f) Species distribution maps of 2 x 1 (binary) preliminary tile interactions.

Cross hatched tiles represent occupancy of both species. See text for details

Table 3.1. Proportion of fungal species *P. placenta*[1], *C. marmorata* & *P. variotii* during preliminary 2 x 1 confronting tessellations. * denotes major differences in outcome between *P. placenta*[1] and *C. marmorata* simultaneously inoculated and pre-colonised tile interactions. ** denotes major differences in interaction rate between *P. variotii* and *C. marmorata* simultaneously inoculated and pre-colonised tile interactions.

Experiment & sampling period (weeks)	**Proportion of fungal species in confronted inoculated tiles**					
Simultaneously -inoculated tiles	**Tile 1**			**Tile 2**		
Cm (Tile 1)		Species			Species	
Pp (Tile 2)	*Pp*[1]	*Cm*	*Pp*[1] & *Cm*	*Pp*[1]	*Cm*	*Pp*[1] & *Cm*
0	0	24	0	24	0	0
2	2	18	4	24	0	0
4	7	15	2	24	0	0
6	**17***	**3***	4	22	1	1
Pv (Tile 1)		Species			Species	
Pp (Tile 2)	*Pp*[1]	*Pv*	*Pp*[1] & *Pv*	*Pp*[1]	*Pv*	*Pp*[1] & *Pv*
0	0	24	0	24	0	0
2	0	21	3	16	0	8
4	0	24	0	21	0	3
6	0	23	1	17	1	6
Cm (Tile 1)		Species			Species	
Pv (Tile 2)	*Pv*	*Cm*	*Pv* & *Cm*	*Pv*	*Cm*	*Pv* & *Cm*
0	0	24	0	24	0	0
2	0	23	1	0	**2****	22
4	0	24	0	0	**5****	19
6	0	20	4	0	**21****	3

Cont...

Pre-colonised tiles		Tile 1			Tile 2		
Cm (Tile 1)		Species			Species		
Pp (Tile 2)		*Pp^{I}*	*Cm*	*Pp^{I} & Cm*	*Pp^{I}*	*Cm*	*Pp^{I} & Cm*
	0	0	24	0	24	0	0
	2	1	18	5	24	0	0
	4	9	13	2	24	0	0
	6	**3***	**16***	5	24	0	0
Pv (Tile 1)		Species			Species		
Pp (Tile 2)		*Pp^{I}*	*Pv*	*Pp^{I} & Pv*	*Pp^{I}*	*Pv*	*Pp^{I} & Pv*
	0	0	24	0	24	0	0
	2	0	24	0	17	0	7
	4	0	20	4	23	0	1
	6	0	24	0	21	0	3
Cm (Tile 1)		Species			Species		
Pv (Tile 2)		*Pv*	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
	0	0	24	0	24	0	0
	2	0	22	2	0	**13****	11
	4	0	24	0	0	**16****	8
	6	0	24	0	0	**20****	4

In addition to the apparent influence of inoculation mode on the outcome of the interactions, the results suggested that three other factors appeared important in determining the presence of a particular species at time of harvest. These were the original presence within the subject tile, pairing combination, and longest sampling period. In order to test the importance of each of these four experimental parameters stepwise logistic regression was used.

Table 3.2. Factors that influenced the presence of a species in a tile, as determined by stepwise logistic regression. (1 = most important; 4 = least important. '-' indicate that the factors were not significant).

Factor	Species					
	Pp[1]	*Cm*	*Pv*	*Pp*[1]*&Cm*	*Pp*[1]*&Pv*	*Cm&Pv*
Presence within subject tile	1	1	1	2	2	2
Pairing combination	2	2	2	1	1	1
Longest sampling period	3	-	3	3	3	3
Inoculation mode	-	3	4	-	-	-

Table 3.2 shows factors that influenced the presence of a species in a tile and their importance as determined by stepwise logistic regression. The model showed that the presence of *P. placenta*[1] depended most importantly on its original presence within the subject tile, of secondary importance was the species with which it was paired, and thirdly the longest sampling period. The mode of inoculation did not significantly influence the outcome of its interactions. For both *C. marmorata* and *P. variotii*, their presence within a tile was dependent on its original presence within the subject tile, the species with which it was paired, and inoculation mode. In addition the longest sampling period was influential for the presence of *P. variotii* within a tile. The models all had highly significant goodness-of-fit statistics ($P<<0.01$ in all cases).

3.3.2. Extended time scale 2x1 simultaneously-inoculated tile interactions

To investigate the reproducibility and the effect of longer incubation times on the outcome of the fungal interactions, simultaneously inoculated 2 x 1 tile interactions between all three species were carried out over an extended time scale of 19 weeks. The outcome interaction results presented in Table 3.3 were compared with the simultaneously inoculated results of the preliminary tile interactions (see Table 3.1) in order to determine the reproducibility of the experimental system. Table 3.4 shows these results expressed as percentages to allow ease of comparison between the two data sets.

In general, the interactions between all three species gave similar final results to the preliminary tile interactions, with the exception of the *P. placenta*^{1} *versus P. variotii* interaction where the final outcome after 19 weeks resulted in the replacement of *P. placenta*^{1} by *P. variotii*. However, data presented in Table 3.4 indicated that the temporal development of the interactions were shown to vary between the two sets of data. This is illustrated in the interaction between *C. marmorata* and *P. variotii* in the extended timescale experiment, where *C. marmorata* replaced *P. variotii* within one week rather as opposed to 4 – 6 weeks during the preliminary interaction study. Also, during the interaction between *P. placenta*^{1} and *C. marmorata*, differences in outcome were observed over the course of the experiment. *C. marmorata* was shown

Table 3.3. Proportion of fungal species *P. placenta*[1], *C. marmorata* & *P. variotii* during extended time scale 2 x 1 confronting tessellations.

Experiment & sampling period (weeks)	Proportion of fungal species in confronted inoculated tiles					
Simultaneously -inoculated tiles	**Tile 1**			**Tile 2**		
Pp (Tile 1)	Species			Species		
Cm (Tile 2)	*Pp*	*Cm*	*Pp & Cm*	*Pp*	*Cm*	*Pp & Cm*
0	40	0	0	0	40	0
1	20	0	20	0	40	0
2	28	0	12	0	40	0
3	36	0	4	0	34	6
5	38	0	2	0	33	7
11	27	5	8	7	12	21
19	37	2	1	32	8	0
Pp (Tile 1)	Species			Species		
Pv (Tile 2)	*Pp*	*Pv*	*Pp & Pv*	*Pp*	*Pv*	*Pp & Pv*
0	40	0	0	0	40	0
1	39	0	1	0	40	0
2	25	0	15	0	40	0
3	39	0	1	0	40	0
5	34	0	6	0	40	0
11	-	-	-	-	-	-
19	0	34	6	0	40	0
Pv (Tile 1)	Species			Species		
Cm (Tile 2)	*Pv*	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
0	40	0	0	0	40	0
1	0	40	0	0	40	0
2	0	40	0	0	40	0
3	0	40	0	0	40	0
5	0	40	0	0	40	0
11	0	40	0	0	40	0
19	0	40	0	0	40	0

Table. 3.4. Comparison between preliminary and extended time scale 2x1 tile interactions expressed as percentages for ease of comparison.

Experiment & sampling period (weeks)	Proportion of fungal species in confronted inoculated tiles (%)					
Preliminary 2x1 tile interactions	**Tile 1**			**Tile 2**		
Pp (Tile 1)		Species			Species	
Cm (Tile 2)	*Pp*	*Cm*	*Pp & Cm*	*Pp*	*Cm*	*Pp & Cm*
0	100	0	0	0	100	0
2	100	0	0	8.33	75	16.67
4	100	0	0	29.16	62.5	8.33
6	91.67	4.16	4.16	70.83	12.5	16.67
Pp (Tile 1)		Species			Species	
Pv (Tile 2)	*Pp*	*Pv*	*Pp & Pv*	*Pp*	*Pv*	*Pp & Pv*
0	100	0	0	0	100	0
2	66.67	0	33.33	0	87.5	12.5
4	87.5	0	12.5	0	100	0
6	70.83	4.16	25	0	83.33	4.16
P v (Tile 1)		Species			Species	
Cm (Tile 2)	*Pv*	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
0	100	0	0	0	100	0
2	0	8.33	91.67	0	95.83	4.16
4	0	20.83	79.16	0	100	0
6	8.33	79.16	12.5	0	83.33	16.67

Cont...

Extended timescale 2x1 tile interactions		Tile 1			Tile 2		
Pp (Tile 1)			Species			Species	
Cm (Tile 2)		*Pp*	*Cm*	*Pp & Cm*	*Pp*	*Cm*	*Pp & Cm*
	0	100	0	0	0	100	0
	1	50	0	50	0	100	0
	2	70	0	30	0	100	0
	3	90	0	10	0	85	15
	5	**95**	**0**	**5**	**0**	**82.5**	**17.5**
	11	67.5	12.5	20	17.5	30	52.5
	19	92.5	5	2.5	80	20	0
Pp (Tile 1)			Species			Species	
Pv (Tile 2)		*Pp*	*Pv*	*Pp & Pv*	*Pp*	*Pv*	*Pp & Pv*
	0	100	0	0	0	100	0
	1	97.5	0	2.5	0	100	0
	2	62.5	0	37.5	0	100	0
	3	97.5	0	2.5	0	100	0
	5	**85**	**0**	**15**	**0**	**100**	**0**
	11	-	-	-	-	-	-
	19	0	85	15	0	100	0
Pv (Tile 1)			Species			Species	
Cm (Tile 2)		*Pv*	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
	0	100	0	0	0	100	0
	1	0	100	0	0	100	0
	2	0	100	0	0	100	0
	3	0	100	0	0	100	0
	5	**0**	**100**	**0**	**0**	**100**	**0**
	11	0	100	0	0	100	0
	19	0	100	0	0	100	0

to initially invade *P. placenta[1]'s* tile after 1 week before *P. placenta[1]* eventually resisted further invasion and invaded *C. marmorata*'s tile after approximately 3 weeks. The data also showed that after 19 weeks the proportion of *C. marmorata* occupying its own resource domain was higher than that shown in the preliminary tile experiment at 6 weeks (20 % compared to 12.5 %, respectively).

3.3.3. Short time scale C. marmorata versus P. variotii simultaneously inoculated tile interactions

The results of the short timescale interactions between *C. marmorata* and *P. variotii* are presented in Table 3.5 These results indicate that *C. marmorata* invaded *P. variotii's* resource domain after 4 days, and thereafter continued to replace *P. variotii.* After 8 days, *C. marmorata* had replaced *P. variotii* in approximately 50 % of *P. variotii's* tile in all 10 replicates. The results of the control experiment suggested that full occupancy of *C. marmorata* in the originally uninoculated tile occurred after 7 days. Comparison of the two data sets may indicate that the presence of *P. variotii* does influence the invasion and colonisation of the subject tile by *C. marmorata.*

3.3.4. Incubation temperature experiment

Visual analysis of the tiles prior to harvest indicated differences in interaction outcome between the three temperature regimes. At 20°C, after 4 days incubation, both *C. marmorata* and *P. variotii* had fully colonised their own tile resource. *C. marmorata's* mycelium was white and woolly in appearance, and advanced approximately 1 mm into *P. variotii's* tile. *P. variotii* was grey-white and downy in appearance with minor production of pale-ochre spores. At 25°C, both species showed full tile growth with *C. marmorata* advancing approximately 4 - 5 mm into *P. variotii's* tile. *P. variotii' s* tile

was orange-brown in colour due to increased spore production. The most noticeable difference between the interactions was seen at 28°C. *P. variotii* displayed prolific sporulation across its own tile and approximately 40 - 70 % (between replicates) of *C. marmorata's* tile. *C. marmorata* showed poor colonisation of its own tile, with only minor production of aerial hyphae from the inoculation core. The interaction outcomes for each temperature regime are shown in Table 3.6 These results indicate that the interaction outcome between *C. marmorata* and *P. variotii* is sensitive to incubation temperature, as high temperatures inhibit the growth of *C. marmorata.*

3.3.5. 'Calibration' binary tile interaction results for large-scale (6 x 6 grid) two species interactions (described in 4.2)

Results of the 2 x 1 binary tile confrontations presented in Table 3.7 showed that *C. marmorata* defended its resource domain against *P. placenta*[2], and invaded and replaced tiles initially occupied by *P. placenta*[2]. *P. placenta*[2] was shown to usually defend its territory against *P. variotii* and *vice versa,* however, 4 out of a total of 24 tiles initially inoculated with *P. placenta*[2] displayed coexistence with *P. variotii. P. variotii* was invaded and subsequently replaced by *C. marmorata,* whilst *C. marmorata* defended its territory against *P. variotii.* The result for *P. placenta*[2] and *C. marmorata* was contary to previous findings presented in 3.1 and 3.2 (where *P. placenta*[2] either replaced *C. marmorata* or there was a deadlock interaction). It was also recorded that *P. placenta*[2] grew as grey-white appressed, submerged mycelium in the interaction with *C. marmorata* and *P. variotii.*

3.3.6. 'Calibration' binary tile interaction results for equal proportion three species interactions

The results of the 2 x 1 binary tile confrontations presented in Table 3.8 showed that *C. marmorata* defended its resource domain against *P. placenta*^{1}, and often invaded tiles initially occupied by *P. placenta*^{1}, however, complete replacement did not occur. *P. placenta*^{1} was shown to usually defend its territory against *P. variotii* and *vice versa*. *P. variotii* was invaded and subsequently replaced by *C. marmorata,* whilst *C. marmorata* defended its territory against *P. variotii.* The result for *P. placenta*^{1} and *C. marmorata* was different to previous findings presented in tables 3.1 and 3.2 (where *P. placenta*^{1} either replaced *C. marmorata* or there was a deadlock interaction) and table 3.8 (where *C. marmorata* replaced *P. placenta*^{2}). The temporal dynamics of the interactions were shown to be consistent with the results shown previously (see 3.3.3 & 3.3.4), with *C. marmorata* replacing *P. variotii* within one week, with the other two confrontations occuring over longer dynamic timescales.

Table 3.5. Proportion of fungal Species *C. marmorata & P. variotii* during short time scale 2 x 1 binary confronting tessellations.

Experiment & sampling period (days)	**Proportion of fungal species in confronted inoculated tiles**					
Simultaneously -inoculated tiles	**Tile 1**			**Tile 2**		
Pv (Tile 1)		Species			Species	
Cm (Tile 2)	*Pv*	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
0	40	0	0	0	40	0
4	32	8	0	0	40	0
6	23	0	17	0	40	0
7	20	20	0	0	40	0
8	19	20	1	0	40	0
Simultaneously -inoculated tiles	**Tile 1**			**Tile 2**		
'Blank' (Tile 1)		Species			Species	
Cm (Tile 2)	'blank'	*Cm*		'blank'	*Cm*	
0	40	0		0	40	
4	20	20		0	40	
6	12	28		0	40	
7	0	40		0	40	
8	0	40		0	40	

Table 3.6. Proportion of fungal species *C. marmorata & P. variotii* during the incubation temperature investigation.

Experiment & sampling period (weeks)	Proportion of fungal species in confronted inoculated tiles					
Incubation temperature 20°C	**Tile 1**			**Tile 2**		
Pv (Tile 1)	Species			Species		
Cm (Tile 2)	*Pv*	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
0	24	0	0	0	24	0
1	0	14	10	0	24	0
Incubation temperature 25°C	**Tile 1**			**Tile 2**		
	Species			Species		
	Pv	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
0	24	0	0	0	24	0
1	12	12	0	0	24	0
Incubation temperature 28°C	**Tile 1**			**Tile 2**		
	Species			Species		
	Pv	*Cm*	*Pv & Cm*	*Pv*	*Cm*	*Pv & Cm*
0	24	0	0	0	24	0
1	24	0	0	3	20	1

Table 3.7. Proportion of fungal species *P. placenta*[2], *C. marmorata,* and *P. variotii* during 'calibration' 2 x 1 confrontations for the large-scale (6 x 6) two species interactions (see 4.2) incubated at ambient temperature.

Sampling period (weeks)	Proportion of fungal species in confronted inoculated tiles					
	Tile 1			Tile 2		
***Pp*[2] (Tile 1)**	Species			Species		
***Cm* (Tile 2)**	*Pp*[2]	*Cm*	*Pp*[2] & *Cm*	*Pp*[2]	*Cm*	*Pp*[2] & *Cm*
0	24	0	0	0	24	0
6	2	18	4	0	24	0
***Pp*[2] (Tile 1)**	Species			Species		
***Pv* (Tile 2)**	*Pp*[2]	*Pv*	*Pp*[2] & *Pv*	*Pp*[2]	*Pv*	*Pp*[2] & *Pv*
0	24	0	0	0	24	0
6	20	0	4	0	24	0
***Cm* (Tile 1)**	Species			Species		
***Pv* (Tile 2)**	*Cm*	*Pv*	*Pv* & *Cm*	*Pv*	*Cm*	*Pv* & *Cm*
0	24	0	0	24	0	0
6	24	0	0	0	24	0

Table 3.8. Proportion of fungal species *P. placenta*[1], *C. marmorata,* and *P. variotii* during 'calibration' 2 x 1 confrontations for the equal proportion large-scale (3 x 3) three species interactions (see 5.3) incubated at 15°C.

Sampling period (weeks)	**Proportion of fungal species in confronted inoculated tiles**					
	Tile 1			**Tile 2**		
***Pp*[1] (Tile 1)**	Species			Species		
***Cm* (Tile 2)**	*Pp*[1]	*Cm*	*Pp*[1] & *Cm*	*Pp*[1]	*Cm*	*Pp*[1] & *Cm*
0	24	0	0	0	24	0
2	23	1	0	0	24	0
4	21	0	3	0	24	0
6	12	0	12	0	24	0
***Pp*[1] (Tile 1)**	Species			Species		
***Pv* (Tile 2)**	*Pp*[1]	*Pv*	*Pp*[1] & *Pv*	*Pp*[1]	*Pv*	*Pp*[1] & *Pv*
0	24	0	0	0	24	0
2	24	0	0	0	24	0
4	24	0	0	0	24	0
6	24	0	2	0	24	0
***Cm* (Tile 1)**	Species			Species		
***Pv* (Tile 2)**	*Cm*	*Pv*	*Pv* & *Cm*	*Pv*	*Cm*	*Pv* & *Cm*
0	24	0	0	24	0	0
2	24	0	0	10	12	2
4	24	0	0	0	24	0
6	24	0	0	0	24	0

3.4. DISCUSSION

The temporal development of the interactions were shown to vary depending on species pairing of the fungi studied. Data presented in Tables 3.1, 3.3 and 3.5, show that the replacement of *C. marmorata* by *P. placenta*^{1} may take as long as 19 weeks. Whereas, the replacement of *P. variotii* by *C. marmorata* took only 1 week (with the exception of the preliminary interaction study were replacement occurred in 6 weeks). These observations are supported by the conclusion of the stepwise logistic regression model, that the effect of longest sampling period on interaction outcome is important for *P. placenta*^{1} but not for *C. marmorata.* During the *P. placenta*^{1} *versus P. variotii* interaction, deadlock was shown after 6 weeks. However, after 19 weeks *P. variotii* had replaced *P. placenta*^{1}. This result may be related to nutrient depletion of the agar tiles, or adverse microenvironment conditions, thus favouring the persistence of *P. variotii.* Variation in the temporal dynamics of individual interactions may be attributed to different growth rates and/or different mechanisms of combat displayed by the three fungi (e.g. hyphal interference, mycelial interactions, and antibiosis). Relatively little is know about the underlying molecular basis of these combat mechanisms, although it is thought that certain genetic regulatory mechanisms and signalling systems may play a role in some interactions, particularly in those displaying co-ordinated developmental shifts (Rayner & Boddy, 1988).

The interaction outcome data can be used to rank the three fungal species on the basis of their combative ability. It was shown that a intransitive hierarchy (Boddy, 2000) was displayed by the fungi studied; *P. placenta*^{1} > *C. marmorata*, *C. marmorata* > *P. variotii*, but *P. placenta*^{1} showed deadlock with *P. variotii.*

This information, if extrapolated to a larger multi-species scenario, might suggest that *C. marmorata* would initially rapidly replace *P. variotii* and then *P. placenta*[1] would replace *C. marmorata* - assuming that all species were randomly distributed and in equal proportions. However, it possible to envisage that the replacement *P. variotii* by *C. marmorata* would increase the occupied domain of *C. marmorata* in a hypothetical multi-species interaction. Therefore it is difficult to predict whether *P. placenta*[1] would replace *C. marmorata* due to a disproportional biomass ratio of the two species. Caution must therefore be taken if using interaction studies to provide the basis of predictive analysis of community structure and development, as the behaviour of individual species must be related to their colonisation strategies as this could significantly influence the activity domain occupied by a species (Rayner & Boddy, 1988). For example, non-combative individuals may often display effective primary resource capture strategies allowing colonisation of a virgin resource before the arrival of any potential competitor species. Whereas, individuals capable of secondary resource capture are typically more combative, resulting in the partial or complete take over of an existing species' domain. The influence of colonisation strategy may explain the differences displayed in interaction outcome between the two inoculation methods investigated in 3.3.1. Simultaneously-inoculated tiles allow an initial period on primary resource capture; therefore, *P. variotii*, that displays a ruderal ecological strategy, colonises the tile relatively quickly and persists longer when confronted by *C. marmorata* compared to the interaction with pre-colonised tiles. However, during the simultaneously-inoculated confrontation between *P. placenta*[1] and *C. marmorata*, *P. placenta*[1] replaces *C. marmorata* even although the growth rate of *P. placenta* is slower. This result would suggest that *P. placenta*[1] is more combative than *C. marmorata* in this confrontation. However, during the confrontation between pre-colonised tiles of these species there is a higher occurrence of deadlock. The reason for such a result

is unclear at present, however, a possible explanation may be related to lower nutrient level in the precolonised tiles (as nutrients will have been depleted during the initial colonisation), therefore the energy available to either species to mount a combat response may be lower compared to that in the simultaneously-inoculated tiles.

Another important consideration when using interaction studies as a tool to predict fungal community development is the reproducibility of interaction outcome between replicates (Boddy, 2000). Variation between replicates was most apparent during interactions incubated under ambient temperatures, compared to the relatively consistent outcomes of the interactions incubated at constant temperatures (Tables 3.6 & 3.8). These results may suggest that fluctuations in the micro - environmental conditions could influence the outcome of individual interactions. In addition, comparison of the *P. placenta*^{1} / *P. placenta*^{2} and *C. marmorata* confrontations (shown in tables 3.1, 3.2, 3.7 and 3.8) can be shown to vary in interaction outcome. The most significant variation is of the *P. placenta*^{2} and *C. marmorata* confrontation where *P. placenta*^{2} is completely replaced. The outcome of replicate fungal interactions may not always the same, even under apparently identical conditions (Boddy, 2000). Indeed, Rayner *et al* (1995) showed that in 20 studied replicates of confrontations between *Peniophora lycii* and *Coriolus versicolor* three different outcomes occurred: *P. lycii* replaced *C. versicolor* in some, *C. versicolor* replaced *P. lycii* in others, and some replicates showed deadlock. However, with the confrontation between *P. placenta*^{2} and *C. marmorata* differences in the morphology of *P. placenta*^{2} were also apparent. These were that the mycelia of *P. placenta*^{2} were appressed and mainly submerged compared to the presence of white cottony aerial mycelia of *P. placenta*^{1}. It is proposed that this morphological change may be related to *P. placenta*^{2}*'s* poor combative ability against *C. marmorata* and this phenomenon is further investigated in Chapter 6.

Indeed, results presented in Chapter 6 identify *P. placenta*^{2} as a morphologically distinct form of *P. placenta*^{1}, hence the adoption of the numeric notation for each *P. placenta* form should be noted in respect to the various interaction outcomes.

In the incubation temperature experiment, the outcome of the interaction between *C. marmorata* and *P. variotii* was found to vary depending on temperature regime. This was most apparent at 28 °C where *C. marmorata* showed poor growth and failed to colonise its own tile resource. Furthermore, *P. variotii* sporulated profusely at 28 °C, which could indicate the operation of a mechanism of survival during stressful environmental conditions. The interaction outcomes at the other two temperatures investigated were found to be similar to that previously reported (*C. marmorata* replaced *P. variotii*), however, the rate of the interaction was found to be slower at low incubation temperatures. These results are supported by those of Magan & Lacey (1984), who found that interaction outcomes did vary with temperature. The effect of the lower incubation could also suggest the differences in interaction outcome of *P. placenta*^{1} and *C. marmorata* during the binary tile experiments at 15 °C (where *C. marmorata* replaced *P. placenta*^{1}) compared to the preliminary binary tile experiments at ambient room temperature (*P. placenta*^{1} replaced *C. marmorata*).

The stepwise logistic regression model for the preliminary binary tile interactions investigated the relative importance of experimental parameters which influenced the eventual outcome of the interactions during the preliminary tile interactions. This information was used to inform the design of subsequent tessellations. Thus, during experiments involving *C. marmorata* and/or *P. variotii* simultaneous inoculation of tiles was important in determining their occupancy on outcome. For example, more tiles

displayed co-existence of the two species during simultaneously inoculated experiments than in pre-inoculated experiments, where *C. marmorata* was shown to replace *P. variotii.* However, neither simultaneous nor pre- inoculation of tiles was important in determining the occupancy of *P. placenta*[1]. Simultaneous inoculation was therefore adopted to avoid rapid extinction of *C. marmorata* and *P. variotii* in further experiments.

In conclusion, this Chapter has demonstrated the use of a novel tessellated agar tile system to study interspecific fungal interactions. It has been shown that interaction outcome is dependent on species pairing, longest sampling period and mode of inoculation. These factors have been used to inform the design of subsequent large-scale tessellations (Chapters 4 & 5). The development of this biological model has key advantages in the study of interspecific fungal interactions. These are namely the incorporation of both spatial and temporal aspects of the heterogeneity displayed in interaction outcome, which may be relevant to the prediction of large-scale multi-species interactions.

CHAPTER 4. LARGE SPATIAL SCALE TWO SPECIES FUNGAL INTERACTIONS

4.1. GENERAL INTRODUCTION

The tessellated agar tile interaction system developed in chapter 3 was shown to allow the effective study of small-scale (binary) two species fungal interactions. However, in the natural environment fungal interactions are considerably more complex, often involving the interaction of numerous species over a range of spatial and temporal scales. Yet, in many studies of wood decay fungal ecology, the results of pairwise plate interactions are still used to explain and sometimes predict fungal community development (e.g. Robinson *et al.* 1993; Rayner & Boddy, 1988, and references therein).

The versatility of the tessellated tile interaction system allows the construction either simple (2 x 1) or complex (n x N) interaction arenas, and therefore has the potential to allow the investigation of spatial scale on the outcome of fungal interactions. The experiments described in this chapter specifically investigate the effect of scaling on interaction outcome, and attempt to determine whether the community dynamics of large-scale two species fungal interactions can be predicted from the outcomes of relevant small-scale binary interactions.

4.2. LARGE SCALE TWO SPECIES 'CHEQUERBOARD' INTERACTIONS

4.2.1. Materials and methods

It should be noted that the morphological distinct form of *P. placenta*^{1} was discovered during this experiment. The confrontations reported in this sub-section therefore refer to *P. placenta*^{2} rather than *P. placenta*^{1}, even though at the time that experiments were conducted it was assumed that *P. placenta*^{1} was being investigated.

Confrontations between *P. placenta*^{2} and *C. marmorata*, *P. placenta*^{2} and *P. variotii*, and *C. marmorata* and *P. variotii* were established by simultaneous inoculation of 6 x 6 agar tile arrays. Each species was inoculated onto alternate tiles to give a 'chequerboard' arrangement, thus giving equal proportions of each species but allowing maximum heterogeneity in spatial distribution of species. Six replicates of each confrontation were constructed and incubated at ambient room temperatures (17 - 25 °C) in the dark. The macro-morphological features of interacting mycelia were recorded over a 6 week period, before final assessment of interaction outcome.

4.2.2. Results

4.2.2.1. Qualitative macro-morphological description of P. placenta^{2}*, C. marmorata and P. variotii interactions for simultaneously inoculated 6 x 6 'chequerboard' tessellated tile interactions.*

(i) *P. placenta*^{2} *vs C. marmorata*: In general, during the interaction between *P. placenta*^{2} and *C. marmorata* both species colonised their respective tiles after approximately 5 – 7 days. *P. placenta*^{2} showed grey-white, appressed,

submerged mycelial growth during colonisation of its tile resource. *C. marmorata* displayed white woolly aerial mycelia that bridged the air-gap between the tiles after approximately 8 – 9 days. Subsequently, the production of yellow interaction lines was observed at the interfaces between *P. placenta*[2] and *C. marmorata* tiles. These lines became dark brown over a period of 2 – 3 weeks. During this time *P. placenta*[2] tiles became dark brown in colour and after approximately 3 – 4 weeks and *C. marmorata* produced a mycelial flush over *P. placenta*[2]*'s* tiles (Figure 4.1a). *C. marmorata* continued to grow over all tiles until time of harvest at 6 weeks.

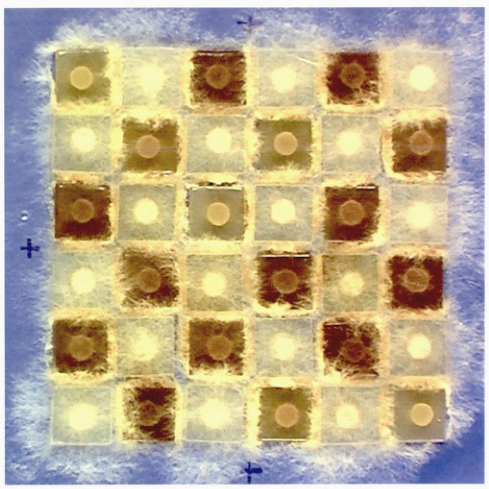
(ii) *P. placenta*[2] *vs P. variotii*: During the interaction between *P. placenta*[2] and *P. variotii* both species colonised their respective tiles after approximately 5 – 7 days. *P. placenta*[2] produced grey-white, appressed, submerged mycelia whereas *P. variotii* grew grey- white downy aerial mycelia turning orange-brown and farinaceous due to the production of spores. A deadlock interaction was shown after approximately 1 week, however, small patches of orange-brown *P. variotii* spores were observed on some *P. placenta*[2] tiles (Figure 4.1b).

(iii) *C. marmorata vs P. variotii*: During the interaction between *C. marmorata* and *P. variotii* interactions, both species colonised their respective tiles after 5 - 7 days. *C. marmorata* subsequently produced a dense mycelial flush over the *P. variotii* tiles, completely engulfing them within 2 weeks (Figure 4.1c).

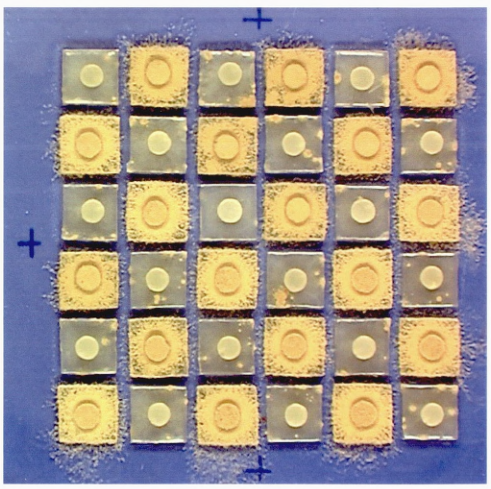
4.2.2.2. Interaction outcomes of 6 x 6 'chequerboard' tessellations.

The outcomes of the 'chequerboard' tile interactions are expressed as species distribution maps and are shown in Figures 4.2. – 4.4. In general, the results reflected the visual observations of the interaction development. For the *P. placenta*^{2} versus *C. marmorata* confrontation, *C. marmorata* was shown to occupy almost all of *P. placenta*^{2} tiles, with only a few domains (29 out of a total 864 tile quarters) of *P. placenta*^{2} remaining at the top left and bottom right of the tessellations. No *P. placenta*^{2} was shown in any tiles initially inoculated with *C. marmorata*. For the *P. placenta*^{2} versus *P. variotii* interaction, each species was found to remain mainly in the tiles that they were originally inoculated onto, with some small patches of *P. variotii* occurring probably due to rapid germination of *P. variotii* spores. For the *C. marmorata* versus *P. variotii* interaction, *C. marmorata* was shown to replace almost all of the *P. variotii* tiles. Indeed, 3 out of the 6 replicates displayed no presence of *P. variotii* at all.

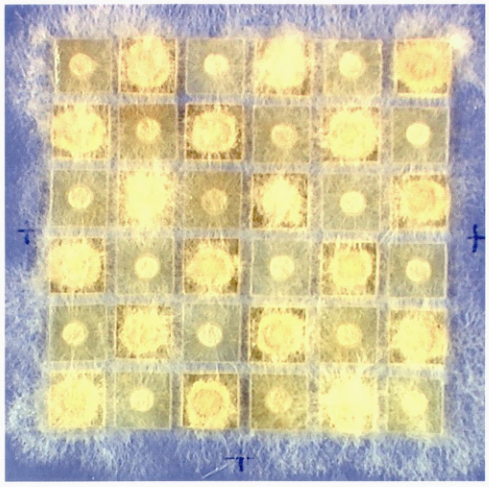
To summarise it was found that during large-scale tile interactions; *C. marmorata* always defended its territory against *P. placenta*^{2} and *P. variotii*, whilst invading *P. placenta*^{2} and *P. variotii's* domains; *P. placenta*^{2} usually defended its territory against *P. variotii* and *vice versa*.



(a)



(b)



(c)

Figure 4.1. Photographs of 6 x 6 grid simultaneously inoculated tile interactions after 6 weeks incubation. (a) *P. placenta*[2] *versus C. marmorata*, (b) *P. placenta*[2] *versus P. variotii*, and (c) *C. marmorata versus P. variotii*. See text for details.

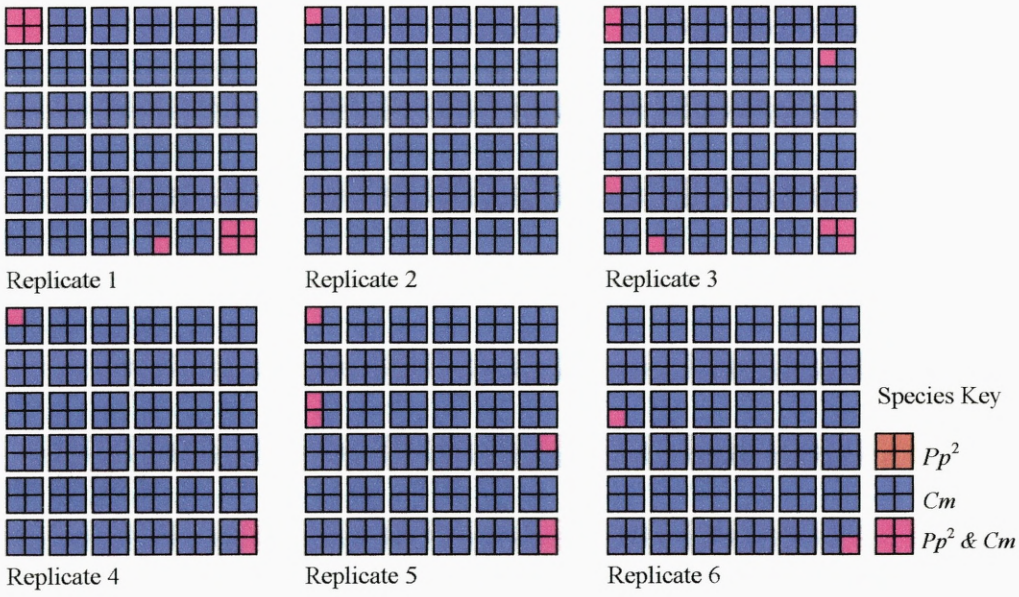


Figure 4.2. Species distibution maps for *P. placenta*[2] versus *C. marmorata* interaction after 6 weeks incubation.

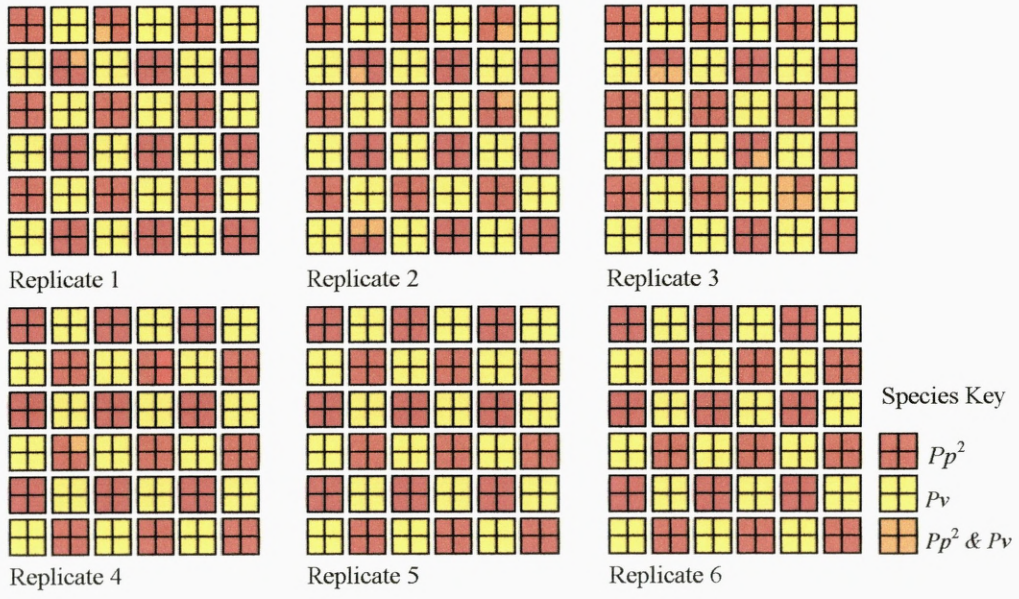


Figure 4.3. Species distibution maps for *P. placenta*[2] versus *P. variotii* interaction after 6 weeks incubation.

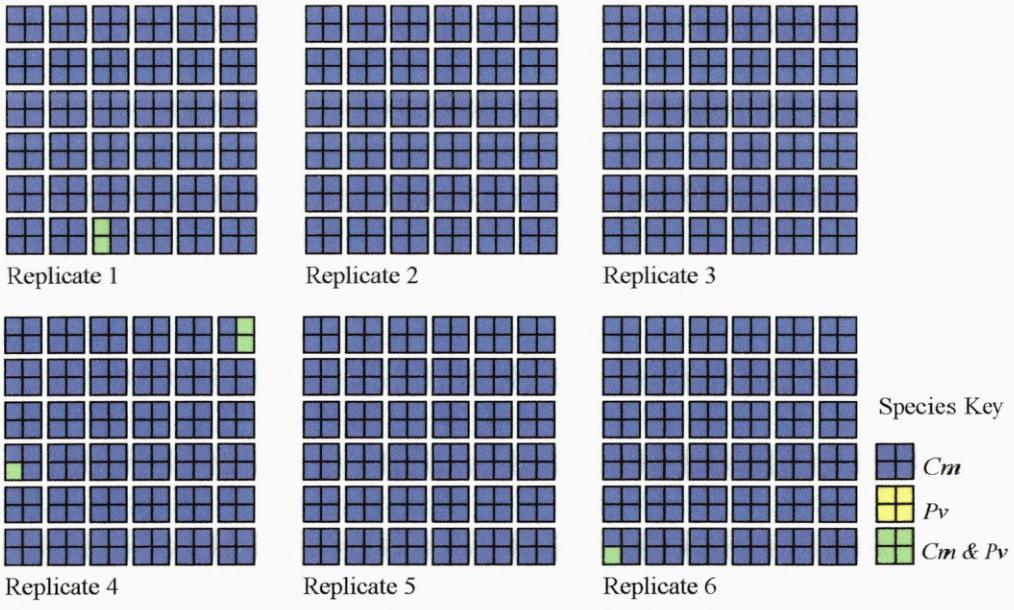


Figure 4.4. Species distibution maps for *C. marmorata* versus *P. variotii* interaction after 6 weeks incubation.

4.2.3. Discussion

Comparison of the large and small scale interaction results indicate that the final outcomes of the large-scale two species interactions can indeed be broadly predicted from the small-scale two species interaction data (2 x 1 interaction data presented in Table 3.7). This agreement between the interaction outcomes at both small and large scales suggest that for two-species interactions of inocula of equal patch size, a bottom up approach for predicting the large-scale behaviour from small scale information is appropriate.

The results of the large-scale interactions also suggest that the number of interaction interfaces may effect interaction outcome. For example, it can be seen that during the interaction between *P. placenta*[2] and *C. marmorata* the majority of *P. placenta*[2] tiles

that were not replaced by *C. marmorata* were at the top and bottom corners of the tessellations. These spatial locations can be seen to possess only two interaction interfaces compared to four that are present in the central area of the tessellation. The lower number of interfaces, and hence the possible number combative interaction fronts, could explain the persistence of *P. placenta*[2] displayed in these spatial locations.

Although it was possible to predict the final outcomes of the large-scale two species interactions, it was not possible to ascertain whether the scaling of the experimental system influenced the temporal dynamics of the interactions as only one destructive sampling was possible after 6 weeks incubation. Therefore, in the subsequent experiment (4.3) the number of sampling periods was increased to facilitate analysis of the temporal aspects of the interaction. In this experiment only *P. variotii* and C. *marmorata* were investigated, as their interaction dynamics were of a more experimentally suitable timescale (i.e. 2 rather than 6 week) so that data could be collected in a shorter time. The incubation temperature was also reduced in an attempt to slow the rate of *C. marmorata's* replacement of *P. variotii.* The subsequent experiment investigates the effect of spatial mixing of species on the temporal dynamics of an interaction between *C. marmorata* and *P. variotii.*

4.3. EFFECT OF SPECIES PATCH SIZE ON THE TEMPORAL DYNAMICS OF AN INTERACTION BETWEEN *C. MARMORATA* AND *P. VARIOTII*

4.3.1. Materials and methods

Large-scale (6x6) tessellations were established between *C. marmorata* and *P. variotii* as described in section 4.2.1. Three different spatial arrangements of species were used to investigate the effect of spatial mixing of species on the dynamics of the community by altering the patch size of species in each tessellation. Therefore, the same-species patch size progressively decreased from tessellation A (patch size 9) to Tessellation C (patch size 1), while the number of inter-species interfaces progressively increased. Ten replicates of each tessellation were incubated at 15 °C in the dark, and 5 replicates were harvested and their interaction outcomes assessed after 8 and 12 days.

4.3.2. Results & discussion

The results presented in figure 4.5 show the spatial distribution of species present within the large-scale *C. marmorata* versus *P. variotii* interactions. In general, it was shown that *C. marmorata* replaced *P. variotii* in all cases, as would be predicted from the relevant binary tile interaction data (described in section 3.3). However, comparison of the spatial distribution of species for each tessellation showed that a greater proportion of *P. variotii* was present in tessellation A (patch size 9) compared to tessellation C (patch size 1) after 8 days incubation. Two possible explanations for these results are that: (a) there is a fundamental difference in the interaction dynamics between the three tessellations (i.e. that species patch size and/or crossing time and/or interface number influence the dynamics); or (b) the differences are trivial (i.e. due to

the greater domain size of *P. variotii* that *C. marmorata* has to replace in tessellation A). Based on spatial distribution data alone, the 'true' explanation of the behaviour shown was not possible to determine.

The application of mathematical based modelling techniques was crucial in distinguishing between the possible explanations described above. The basis behind the mathematical approach adopted in this study involved the use of the *C. marmorata* versus *P. variotii* binary tile interaction data to define a set of rules to calibrate a cellular automaton based computer model (Bown *et al.*, 1999). A cellular automaton model consists of individual cells that are usually arranged in a regular lattice. The evolution of each cell within the lattice is determined by the states of the surrounding cell neighbourhood, based on the calibration rules. The results of the simulated interaction can then be compared to the experimental results. In this study, the cellular automaton was used to specifically test whether the large-scale dynamics could be explained by independent spatially localised interactions (i.e. that individual nearest-neighbour challenges are independent). The predicted results of the model did not agree with the experimental results (data not shown) (Bown, *et al.*, 1999). This finding is important as it demonstrates that the large-scale dynamics of the tessellations do not result from independent nearest neighbour interactions.

The model was then used to test a second hypothesis that, the outcome of a five-cell neighbourhood can be expressed as the weighed sum of pairwise challenges. The results presented in Table 4.1 show the comparison between the experimental and simulated mean state transition data for each tessellation. Note that the transitions *C. marmorata* $\Rightarrow$ *P. variotii* and *C. marmorata* $\Rightarrow$ *C. marmorata:P. variotii* were never

observed and thus have zero frequency. Similarly, *C. marmorata* ⇒ *C. marmorata* was always observed and thus it is assumed to have unitary probability. These transitions were omitted from the tables for sake of clarity. The simulated results were shown to have good agreement with the biological data for all tessellations after 8 days incubation (i.e. all observed state transition data were within the 95 % confidence intervals). However, after 12 days incubation, only tessellation A was shown to display good agreement between biological and simulated data. To investigate the poorer agreement shown at the 12 day incubation period in tessellation's B and C, the effect of patch size on the local interactions was considered. This was done by altering the equations used within the model, to essentially weaken the challenge of *C. marmorata* on *P. variotii* as patch size decreased. These alterations were shown to significantly improve the model, and suggest the large-scale patterning of species (i.e. patch size) influence the temporal dynamics of the interaction. This result is also in agreement with both experimental and theoretical studies that have shown that the degree to which organisms are spatially mixed greatly influences the dynamics of the system (Tilman & Kareiva, 1997).

This study has shown that the prediction of the final interaction outcome for the large-scale confrontations was possible from the small-scale binary confrontations. However, explanation of the underlying dynamics shown in the large-scale interactions was not possible. However, by the use of mathematical based modelling it was possible to test various assumptions regarding the interactions. Specifically, the mathematical modelling approach showed that the large-scale dynamics were not a result of independent, local-neighbourhood challenges and that the community-scale dynamics in the experimental system are therefore a consequence of non-independent local

interactions. This result suggests the interaction dynamics are modified by the community-scale features of the system. The biological basis of this modification may be attributed to phenomena such as anastomosis, which may lead to the translocation of resources from sites of low combative stress to interaction fronts where antagonistic or defence responses increase metabolic demand (Bown, *et al.*, 1999).

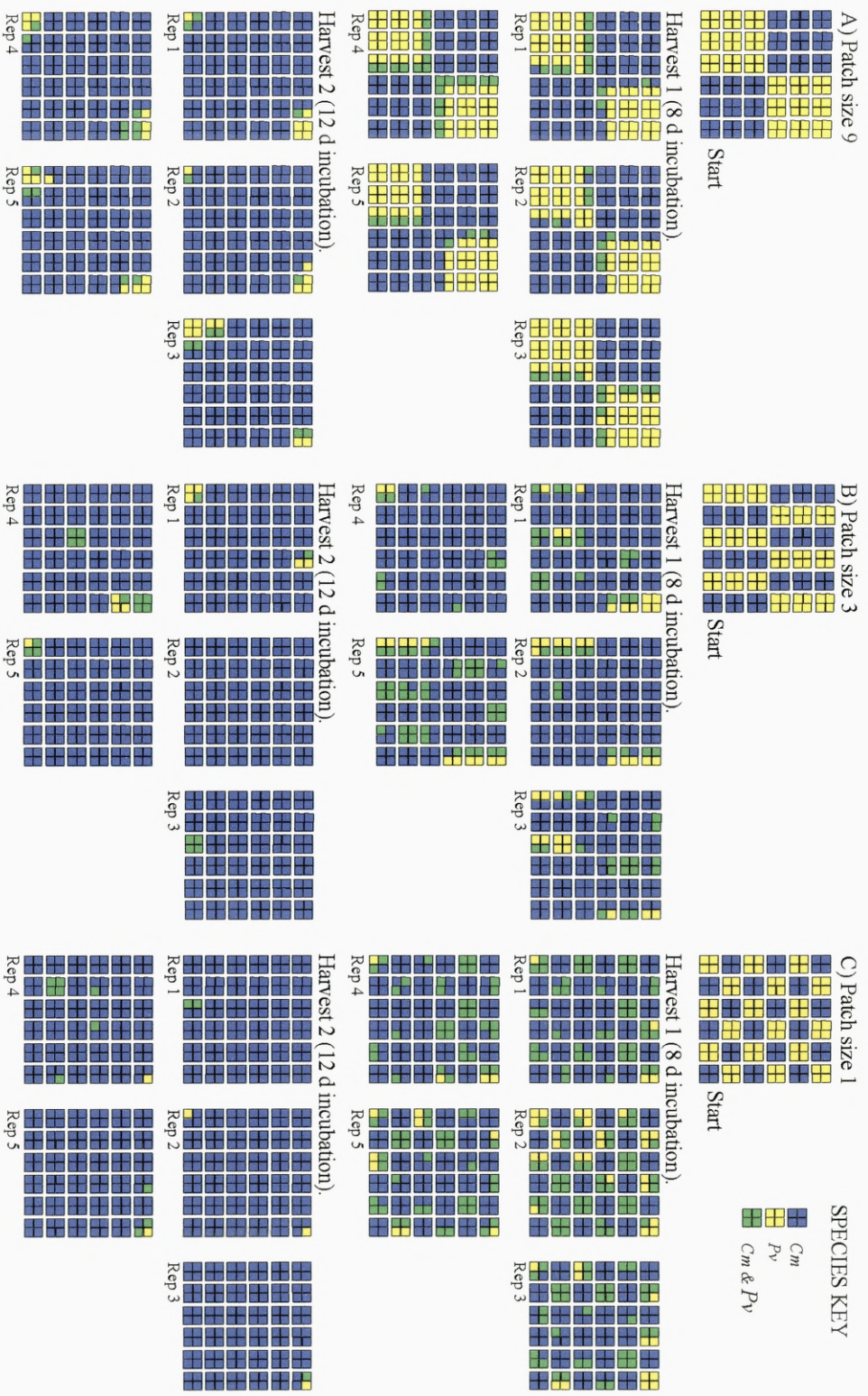


Figure 4.5. Distribution of fungal species extant within varying patch size tile interactions between *C. marmorata* and *P. variotii* after 0, 8 and 12 days incubation at 15 °C.

Table 4.1. Results of the comparison between the experimental and simulated model output for large- scale *C. marmorata versus P. variotii* interactions. Table shows the observed state transition data corresponding to the number of each type of transition averaged over five replicate experiments at day 8 and 12. Subscript (b) & (s) denote biological and simulated data, respectively. Numbers in brackets represent the 95 % confidence interval (lower, upper) derived from the simulations, and the emboldened transition data denote the optimal fit to the observed data (Bown, *et al.*, 1999).

		State transition categories		
Tessellation	Days	*Pv⇒Pv*	*Pv⇒Cm*	*Pv⇒CmPv*
A_b	8	8.6	0	9.4
A_s	8	9.37 (7,13)	0.09 (0,1)	8.06 (5,11)
A_b	12	0.4	14	3.6
A_s	12	2.79 (0,4)	7.13 (4,12)	8.07 (4,11)
B_b	8	0.4	9.2	8.4
B_s	8	0.34 (0,2)	6.22 (3,11)	11.43 (7,15)
B_b	12	0	16.6	1.4
B_s	12	0 (0,0)	17.16 (15,18)	0.83 (0,3)
C_b	8	**0.2**	**1**	**16.8**
C_s	8	**0.88 (0,3)**	**0.69 (0,3)**	**16.42 (14, 18)**
C_b	12	**0**	**15.8**	**2.2**
C_s	12	**0 (0,0)**	**15.57 (14,18)**	**2.43 (0,5)**

4.4. DEVELOPMENT OF INTERACTION ZONE LINES IN 'CHEQUERBOARD' TESSELLATION INTERACTIONS BETWEEN *P. PLACENTA*[1] AND *C. MARMORATA*

4.4.1. Introduction

Interaction zone lines are often clearly observable as narrow, dark lines within cross sections of colonised timber, where the mycelia of two genetically different fungi come into contact (see example of timber cross section (Fig. 4.6.)). These lines are composed of much branched, often melanized hyphae with individual compartments being either inflated or distorted into a variety of shapes (Rayner & Boddy, 1988). The resulting dense mass of mycelial tissue forms a resistant barrier to both mycelial and fluid penetration and is therefore of considerable importance to the defence of captured resource domains.

The interaction between *P. placenta*[1] and *C. marmorata* during large-scale tile interactions provides an excellent opportunity to study interaction zone line formation in a 2-dimensional manner (as opposed to the 1-dimensional manner of the binary tile interactions). The subsequent experiment was designed to study the temporal nature of interaction zone line formation and their possible role in the development of the community. Additionally, the experiment investigated the effect of any diffusible compounds produced by either species during the interaction, using tessellations with or without air-gaps between the tiles. Such diffusible compounds could be antagonistic in nature and significantly influence the interaction dynamics. In experiments reported in previous sections, air-gaps were intentionally included in experimental studies to restrict the diffusion any such compounds in an attempt to limit the number of experimental parameters influencing interaction development within the tessellated

agar tile interaction system.

4.4.2. Materials and methods

Confrontations were established between *P. placenta*[1] and *C. marmorata* using simultaneously inoculated tiles. Two 6 x 6 tessellation types were used to investigate the effect of diffusible compounds. Tessellation A consisted of tiles separated by air gaps, as in section 4.2.1. and tessellation B consisted of tiles with no air gaps. Five replicates of each tessellation were incubated at ambient temperatures (20 – 25 °C) in the dark for 6 weeks. A representative tessellation was photographed every 2 days using Quantimet 600 image analysis software to record the development of the interaction. Tiles were assessed for interaction outcome after 6 weeks using the method described in section 2.4.1.

4.4.3. Results

4.4.3.1. Visual assessment of interaction front development

(i) In general, during the interaction between *P. placenta*[1] and *C. marmorata* in tessellation A, each species colonised their respective tiles after approximately 5 – 7 days. Subsequently, yellow interaction zone lines were formed in the air gaps between the tiles where the mycelium of each species met after 7 – 9 days (see arrows in Figure 4.7a). The spatial positioning of the lines were initially at the edge of the *P. placenta*[1] tiles. This effect was most probably due to the faster growth rate of *C. marmorata.* Over the following 2 week period the lines changed from yellow to orange and finally to a dark brown colour. During this time it was observed that *P. placenta*[1] produced a mycelial flush over the interaction zone line. In association with these events, several of *C. marmorata*

tiles became brown in colour (Figure 4.7b). *C. marmorata's* tiles continued to darken in colour as *P. placenta*[1] grew over all *C. marmorata* tiles (Figures 4.7c & d) until time of harvest at 6 weeks.

(ii) Initially, during the interaction between *P. placenta*[1] and *C. marmorata* in tessellation B, *C. marmorata* colonised a greater resource area compared to *P. placenta*[1] due to *C. marmorata's* faster growth rate. Subsequently, after approximately 5 – 7 days the colony diameters of each individual species ranged from 12 – 15 mm for *C. marmorata* and between 5 – 8 mm for *P. placenta*[1]. Where the mycelia of *C. marmorata* and *P. placenta*[1] met, yellow circular interaction lines were produced (see arrows in Figure 4.8a). During the following 7 – 10 days (Figure 4.8b & c), these interaction lines were observed to gradually increase in size as they advanced into *C. marmorata's* tiles. After approximately 3 weeks the interaction zone lines were difficult to observe as the entire agar slab was covered in an indistinguishable mass of yellow-brown mycelium (Figure 4.8d). The pigmentation of the entire agar slab during the interaction was shown to change from the initial transparent hue to a dark brown colour during the course of the interaction

4.4.3.2. Assessment of interaction outcome

Results presented in Table 4.2. show the mean state transition of species in each tessellation after 6 weeks incubation. In general, the results indicated that in both tessellation types *P. placenta*[1] replaced *C. marmorata*. However, it was apparent that tessellation A displayed a small amount of co-existence between *P. placenta*[1] and *C. marmorata* compared to tessellation B. This difference could

be attributed to the differences in tessellation set-up. The interaction outcome results shown in this study were generally in agreement with predictions of large scale two species community development derived for the relevant binary tile interaction data described in section 3.3.

4.4.4. Discussion

Comparison of the visual analysis results between the two tessellation types indicated differences in interaction development. The most distinct difference was shown in tessellation A where only tiles initially inoculated with *C. marmorata* developed the brown colouration; whereas, in tessellation B the entire agar slab changed colour. This observation could suggest that as *P. placenta*^{1} invaded *C. marmorata's* tiles in tessellation A, some feature of the interaction resulted in the development of the brown pigmentation, however, due to the presence of the air gaps the diffusion of the pigment was restricted. Furthermore, the lack of pigment production in *P. placenta*^{1} tiles could suggest that the interaction zone lines act as a physical barrier to mycelial invasion, and therefore no mycelial interaction is possible between *P. placenta*^{1} and *C. marmorata* within *P. placenta*^{1}*'s* tiles. However, such a barrier may only be beneficial to a species if the species has an available mode of exit from the resource unit. Possible exit modes could be through the production of spores, which have the ability to persist until more favourable conditions arise, or through the production of structures bringing about the invasion and replacement of the confronting species (mycelial cords). The interaction outcome results indicated that in both tessellation types *P. placenta*^{1} replaced *C. marmorata* suggesting that the latter exit mode was displayed.

Another difference displayed between the two tessellation types was the observation of the interaction zone lines as rings in tessellation B. This effect was most likely because each species grew radially without being restricted to their individual tiles. It is possible that the presence of air-gaps in tessellation A could impose stress on the species thereby favouring colonisation of its tile first before bridging the air gap in search of new resources.

The presence of air gaps within tessellation A are a highly unnatural feature of the experimental system and may be involved in the differences in interaction outcome between the two tessellation types. This feature of the experimental system should therefore be carefully considered if community development predictions of real life situations are to be made from this experimental approach.

The use of image analysis techniques in this experiment was useful in recording the development of the interaction zone lines formed between *P. placenta*[1] and *C. marmorata*. Indeed, this method was effective in recording the increase in size of the interaction zone as *P. placenta*[1] replaced *C. marmorata*, and stresses the dynamic aspect to interaction zone line formation. However, the visual observation results suggest that difficulties exist in determining which species are present in the microcosms, particularly after long incubation times. Therefore, the use of re-isolation techniques are still valuable in interaction outcome assessment.

Figure 4.6. Cross section of a Scots Pine (*Pinus sylvestris*) log showing presence of interaction zone lines where mycelia of two (or more) genetically different fungi have come into contact (indicated by white arrows).

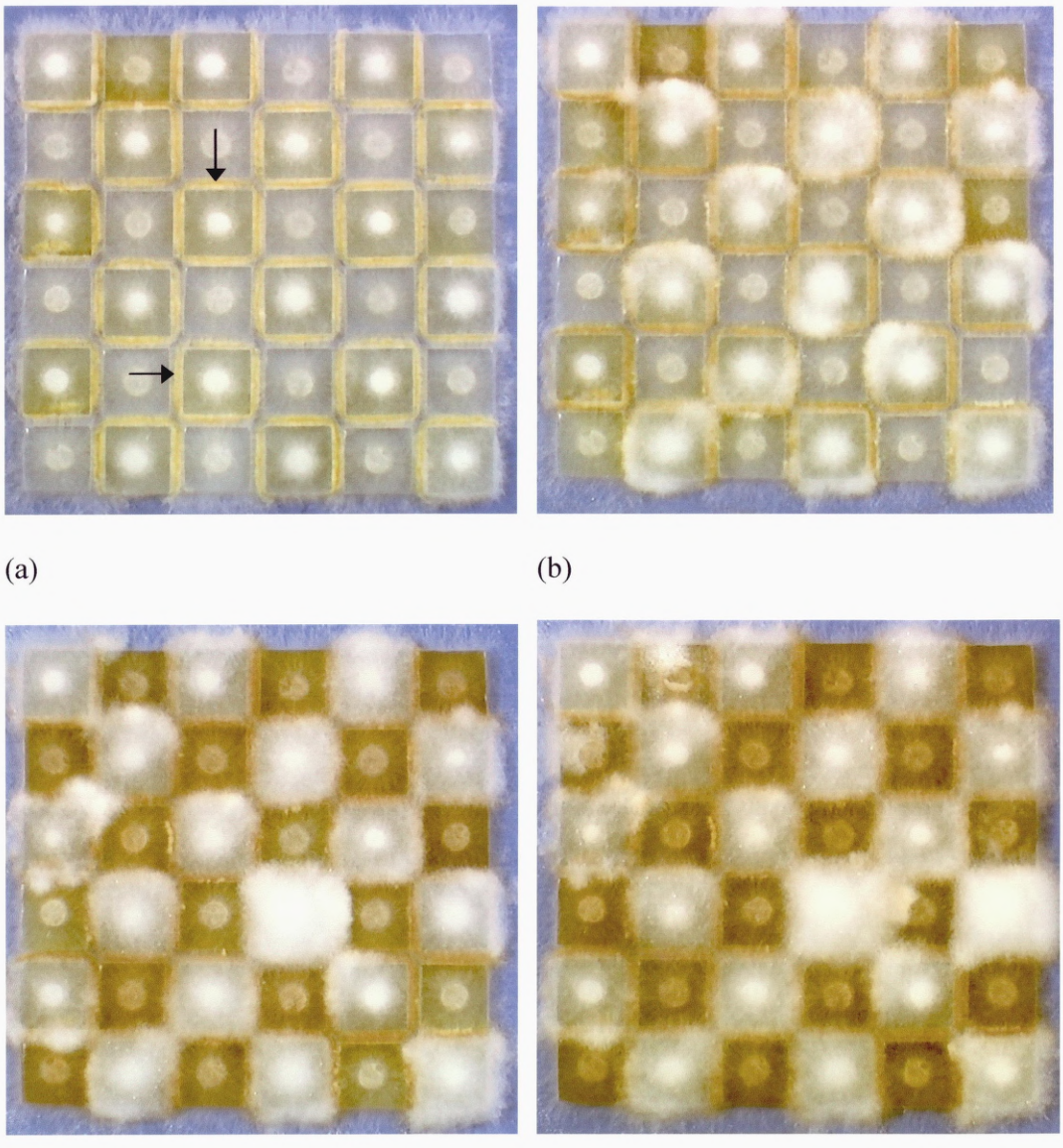
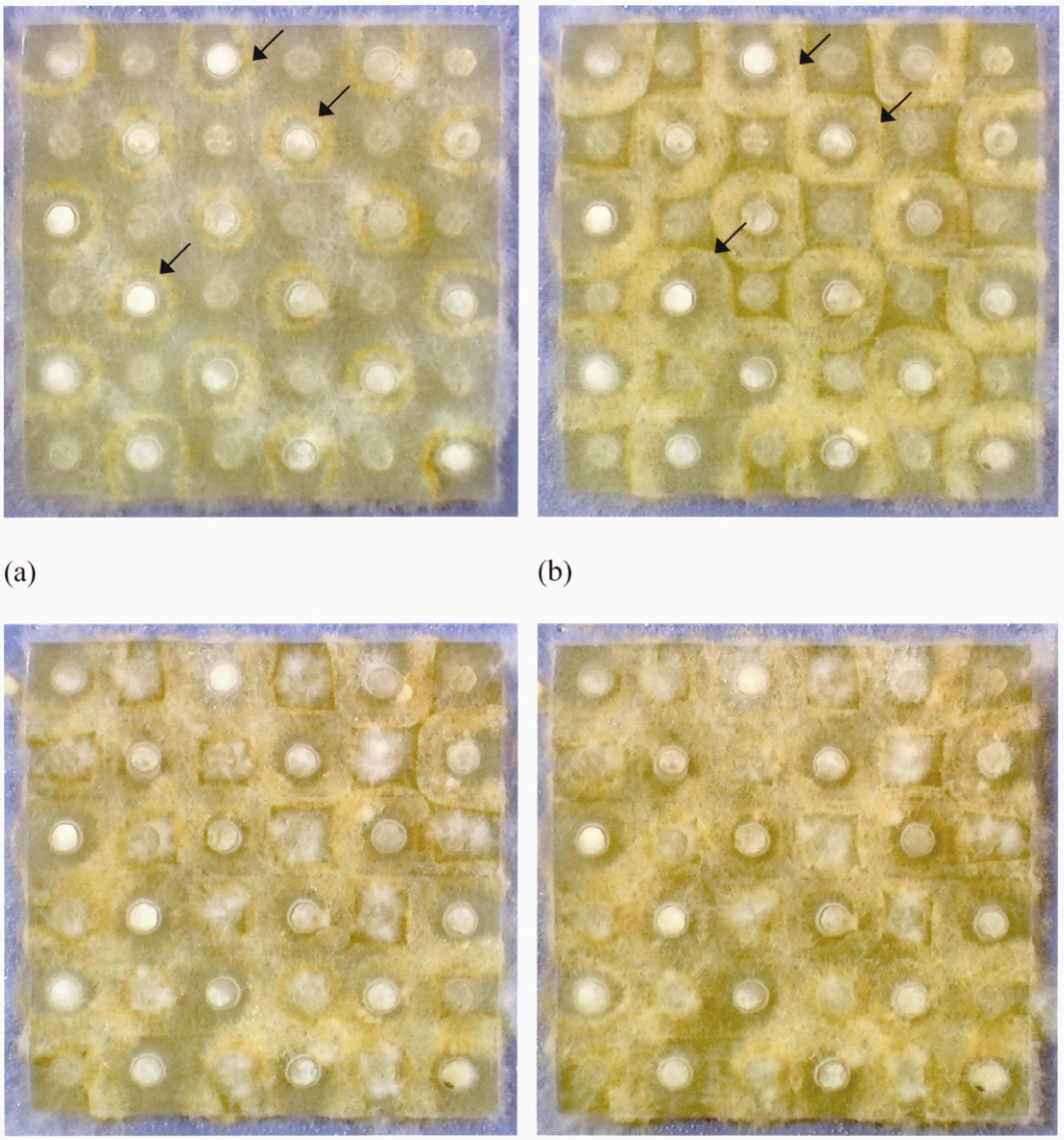


Figure 4.7. Interaction between *P. placenta*[1] and *C. marmorata* in 'Chequerboard' tessellation with air gaps after (a) 9 days, (b) 11 days, (c) 15 days and (d) 20 days incubation. Arrows indicate formation of interaction zone lines. See text for details.



(a) (b)

(c) (d)

Figure 4.8. Interaction between *P. plaenta*[1] and *C. marmorata* in 'Chequerboard' tessellation without air gaps after (a) 9 days, (b) 11 days, (c) 15 days and (d) 20 days. Formation of interaction zone lines indicated by arrows. See text for details.

Table 4.2. State transition data for large-scale *P. placenta*^{1} *versus C. marmorata* interactions.

		State transition categories					
Tessellation	Time (weeks)	*Pp*^{1}>*Pp*^{1}	*Pp*^{1}>*Cm*	*Pp*^{1}>*Pp*^{1}*Cm*	*Cm*>*Cm*	*Cm*>*Pp*^{1}	*Cm*>*CmPp*^{1}
A&B	0	18	0	0	18	0	0
A	6	17.6	0	0.4	0	17.1	0.9
B	6	18	0	0	0	18	0

4.5. CONCLUSIONS

Overall, this chapter has shown that although the small-scale interaction data can be used to predict the final outcome of the large-scale interactions, it is impossible to determine the exact temporal dynamics without aid of mathematical based modelling techniques. These techniques also suggest that large-scale behaviour can be influenced by ecologically adaptive strategies, such as hyphal networking, and nutrient translocation. Moreover, the effect of scale has significant implications on how communities are studied, and therefore must incorporate any emergent behaviour resulting from the collective response of individual processes. This chapter has also shown the application of the tessellated agar tile interaction system to study the formation of interaction zone lines between *P. placenta*^{1} and *C. marmorata*. These studies have highlighted the possible role of interaction zone lines and the effect of diffusible compounds during the interaction on the structure and development of the fungal community. The modelling work presented in this chapter was carried out by James Bown a PhD mathematical modelling and computing student.

CHAPTER 5. THREE SPECIES FUNGAL INTERACTIONS

5.1. GENERAL INTRODUCTION

The experiments presented in this chapter investigate the development of three species fungal interactions in both small-scale (3 x 3) and large-scale (6 x 6) tessellated agar tile interactions. Experimental evidence reported in Chapter 4 demonstrated that the interaction outcomes of large-scale two species interactions were predictable using small-scale, pairwise interaction outcome data even although the exact temporal dynamics of the large-scale interactions were more difficult to elucidate. Very little work has been carried out on multi-species fungal interactions *in vitro*, and it is possible that the introduction of a third species may influence the behaviour of two species interactions, thus influencing the development of the community.

This Chapter specifically assesses the use of species combative hierarchy information (derived from the pairwise tile interactions in chapter 3) to predict the outcome of the large-scale three species interactions. Furthermore, the effects of spatial arrangement and proportion of species on community development will also be investigated.

5.2. SMALL-SCALE (3 x 3) & LARGE-SCALE (6 x 6) THREE SPECIES INTERACTIONS (UNEQUAL INOCULUM PROPORTION)

5.2.1. Materials and methods

5.2.1.1. Small-scale 3 x 3 tessellations

Simultaneously inoculated tiles of *P. placenta*[1], *C. marmorata*, and *P. variotii* were confronted in 10 randomly generated starting configurations (labelled M, N, O, P, Q, S, T, U, W, and X) all in the inoculum ratio of 1: 4: 4 respectively. The starting configurations were created using a random number generator computer program (B. Samson, personal communication). The starting layouts for these tessellations are shown in Figure 5.1. The 1: 4: 4 species inoculation ratio was used because the initial binary tile interactions indicated that *P. placenta*[1] was the most combative species. It was hypothesised that to provide dynamic interactions over experimentally reasonable time scales the initial presence of *P. placenta*[1] be kept to a minimum. Five replicates of each tessellation were constructed and incubated at ambient temperatures in the dark. Interaction outcomes were assessed after 7 weeks.

5.2.1.2. Large-scale 6 x 6 tessellations

Simultaneously inoculated tiles of *P. placenta*[1], *C. marmorata*, and *P. variotii* were confronted in 4 randomly generated starting configurations (labelled G, J, A, and K) with the species inoculum ratio of 1: 4: 4, respectively. The starting layouts for these tessellations are shown in Figure 5.2. Five replicates of each tessellation were constructed and incubated at ambient temperatures in the dark.

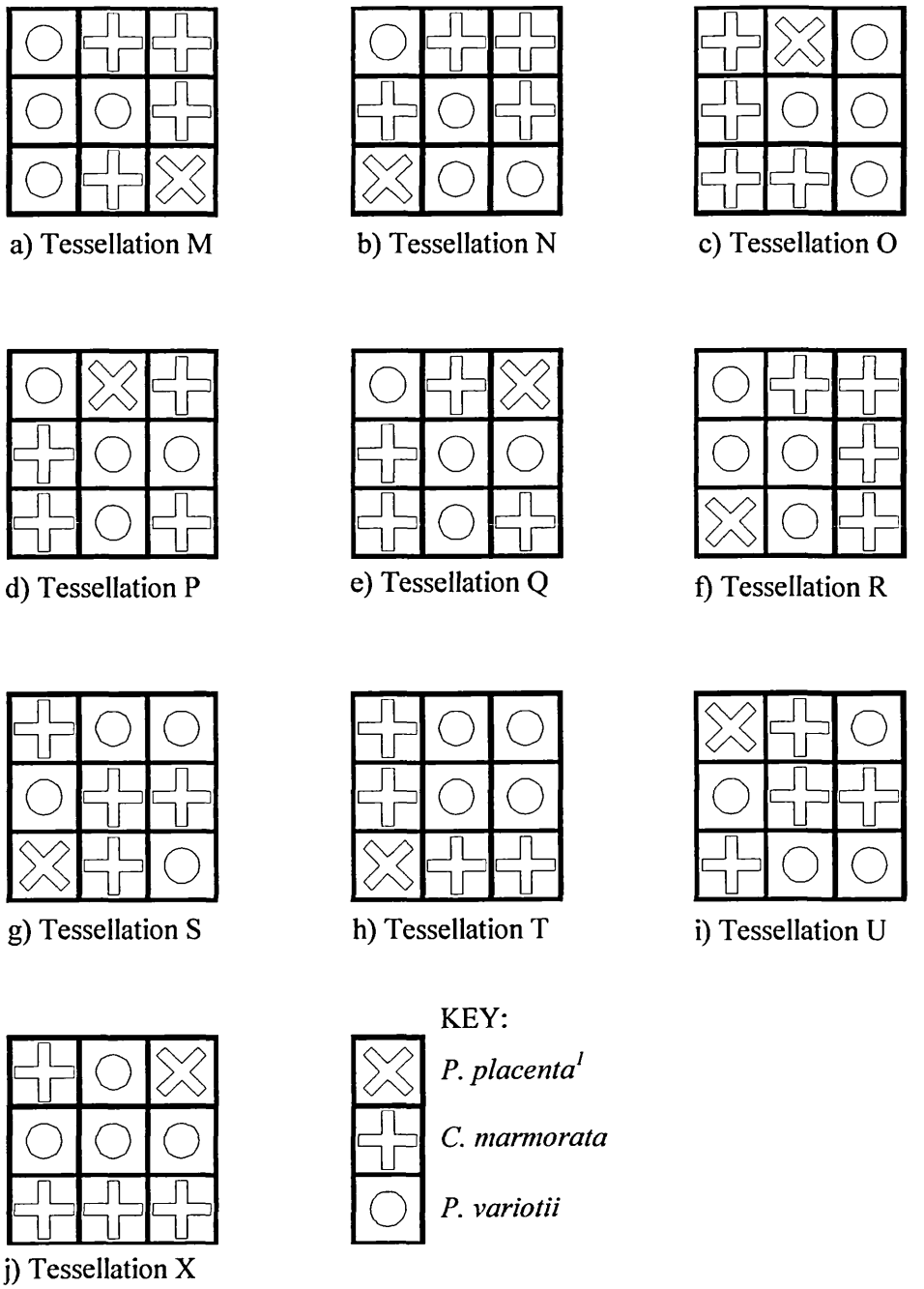


Figure 5.1. Example maps showing the spatial distribution of fungal species in the 3 x 3 tessellated agar tile arrays (1:4:4 inoculation ratio) at the onset of the experiment. Bold lines denote air-gaps between individual tiles. (a – j) Tessellations M; N; O; P; Q; R; S; T; U; and X, respectively. Dimension of each tile is 1 cm^2. Symbols indicate the species inoculated onto each tile.

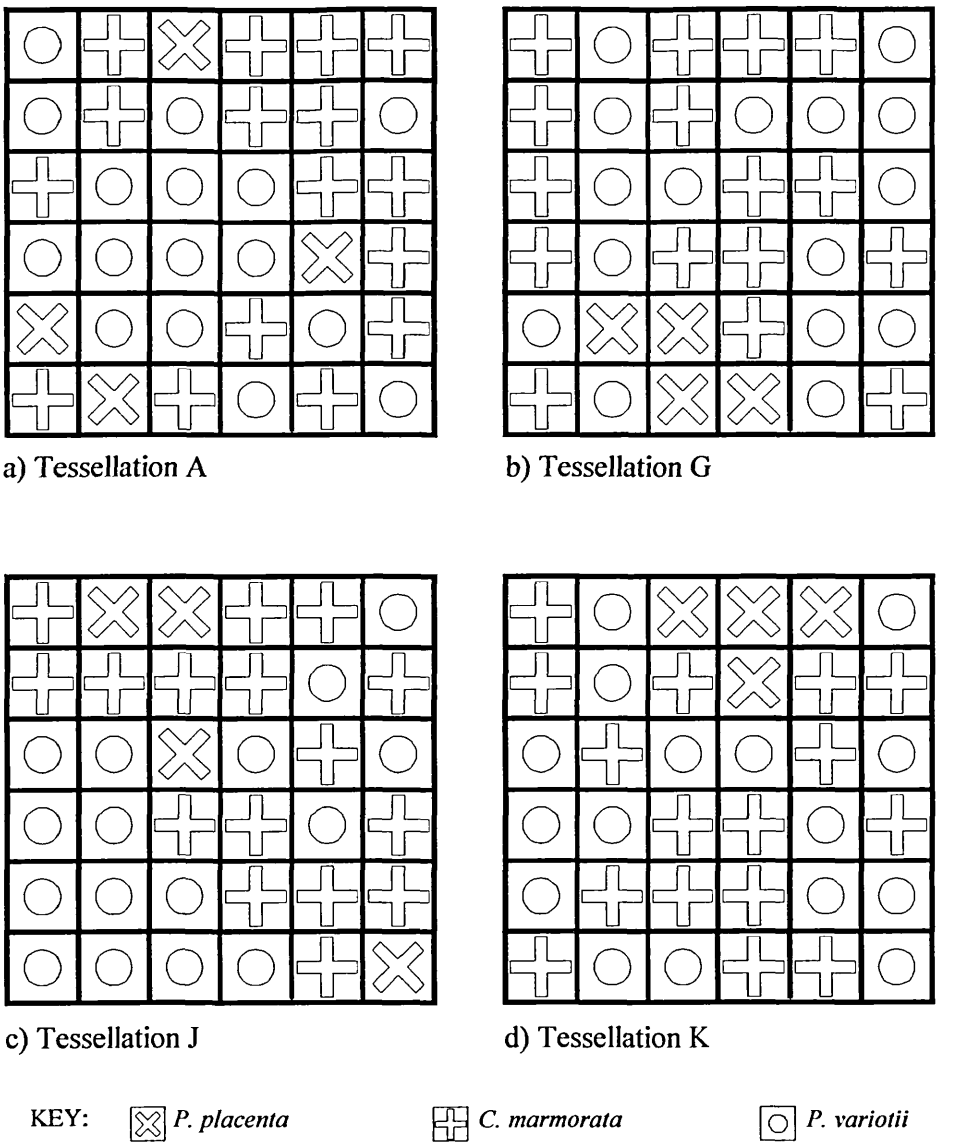


Figure 5.2. Example maps showing the spatial distribution of fungal species in the 6 x 6 tessellated agar tile arrays (1:4:4 inoculation ratio) at the onset of the experiments. Bold lines denote air-gaps between tiles. (a) Tessellation A; (b) Tessellation G; (c) Tessellation J; (d) Tessellation K. Dimension of each tile is 1 cm^2. Symbols indicate the species inoculated onto each tile. Interaction outcomes were assessed after 7 (Tessellations G & J) and 28 (Tessellations A & K) weeks.

5.2.2. Results

5.2.2.1. Small-scale 3 x 3 tessellations

Visual analysis of the 3 x 3 tessellations before assessment of interaction outcome indicated that a variety of spatial patterns were apparent both between individual replicates and tessellations (Figure 5.3). This heterogeneity was confirmed in the interaction outcome data, represented as species spatial distribution maps shown in Figure 5.4. In most of the tessellations, *C. marmorata* appeared to be the most dominant species with the exception of tessellation Q, where *P. variotii* occupied the majority of tiles in all five replicates. Furthermore, it was apparent that 27 (out of 50) of tiles initially inoculated with *P. placenta*[1] had changed state to contain either *P. variotii* alone or in co-existence with *C. marmorata.*

The interaction outcomes were shown to be intuitively concordant with the clustering and scattering of 1st and 2nd principal components for the 3 x 3 data (open symbols) shown in Figure 5.5.

5.2.2.2. Large-scale 6 x 6 tessellations

As in the 3 x 3 tessellations, a variety of spatial patterns in species occupancy both between tessellations and individual replicates were shown in the visual observation of the 6 x 6 tessellations before harvest (see Figure 5.6). This heterogeneity was confirmed by the interaction outcome data, represented as species spatial distribution maps shown in Figure 5.7. Due to the variation in interaction outcome it was therefore difficult to assess any differences between

the two harvest times. However, it was apparent that after 7 weeks incubation, *P. placenta*[1] was still present in 9 out of the 10 replicates over both tessellations. Yet, in tessellations A & K (harvested after 28 weeks) the presence of *P. placenta*[1] was low and a high occupation of *C. marmorata* & *P. variotii* was displayed. Again, as was shown in the 3 x 3 tessellations, the variation observed in species patterning was in agreement with the clustering and scattering of 1st and 2nd principal components shown in Figure 5.5 (closed symbols).

5.2.3. Discussion

In section 3.4, a 'intransitive hierarchy' of combative ability was proposed for the three species investigated in this study. To summarise, it was predicted that in a multi-species interaction scenario *C. marmorata* would rapidly replace *P. variotii*, then *P. placenta*[1] would replace *C. marmorata*. However, results of these three species interactions displayed variable interaction outcomes predominated mainly by the occurrence of *C. marmorata* and *P. variotii*. These data suggest that prediction of multi-species interactions extrapolated from the outcome pairwise interaction studies must therefore be made with caution. In particular, the predicted rapid extinction of *P. variotii* was not shown and the occurrence of *P. placenta*[1] was much lower than predicted. The apparent persistence of *P. variotii* may simply be a reflection of the selectivity of outcome determination method, and that *P. variotii* spores were rapidly germinating on contact with the fresh nutrient agar. Such behaviour is characteristic of the ruderal ecological strategy displayed by *P. variotii*, which tend to result in

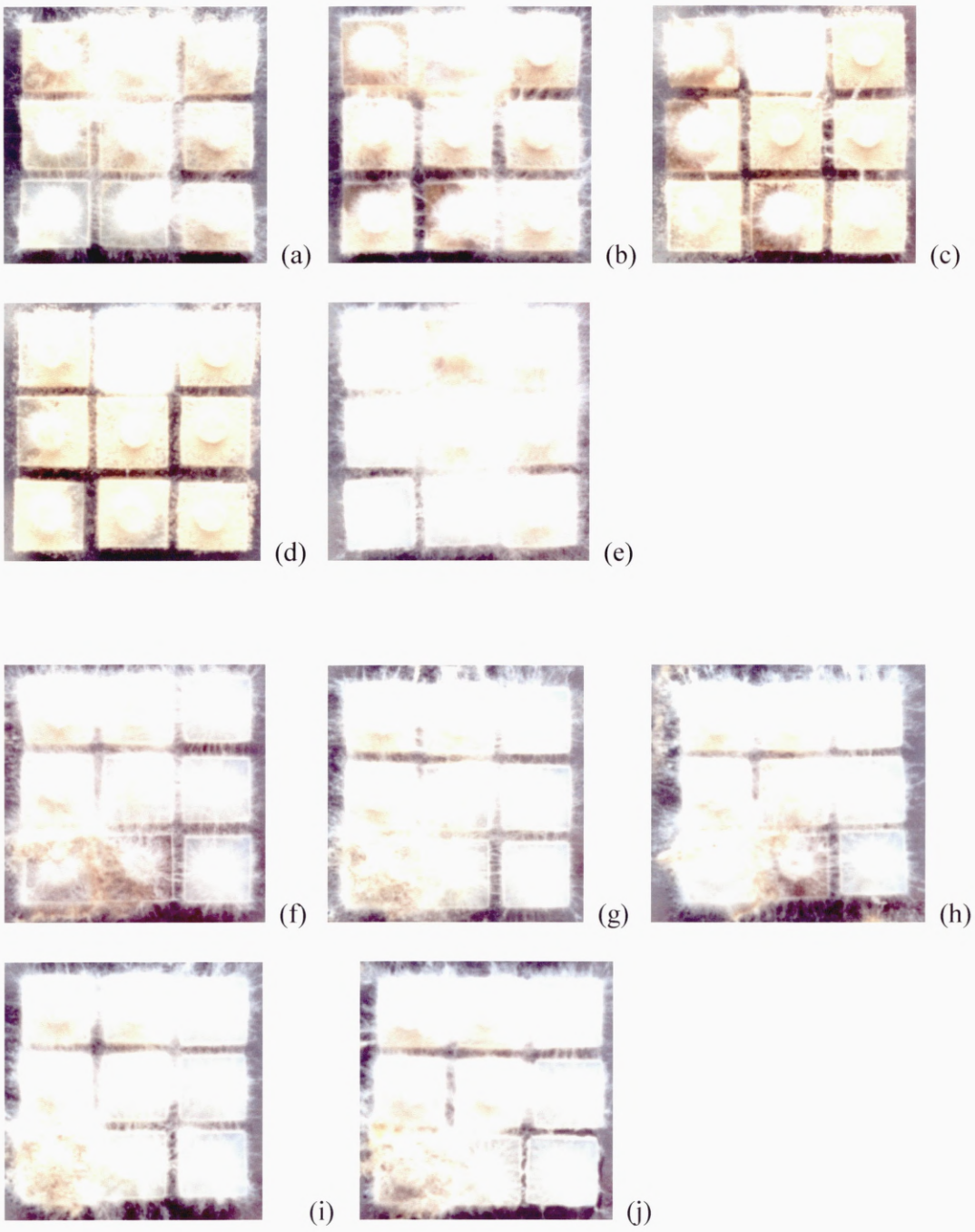


Figure 5.3. Example photographs of 3 x 3 grid, three species tile interactions (Tessellations O & S) after 7 weeks incubation at ambient temperatures. (a – e) Tessellation O, replicates 1 – 5, respectively; (f – j) Tessellation S, replicates 1 – 5, respectively.

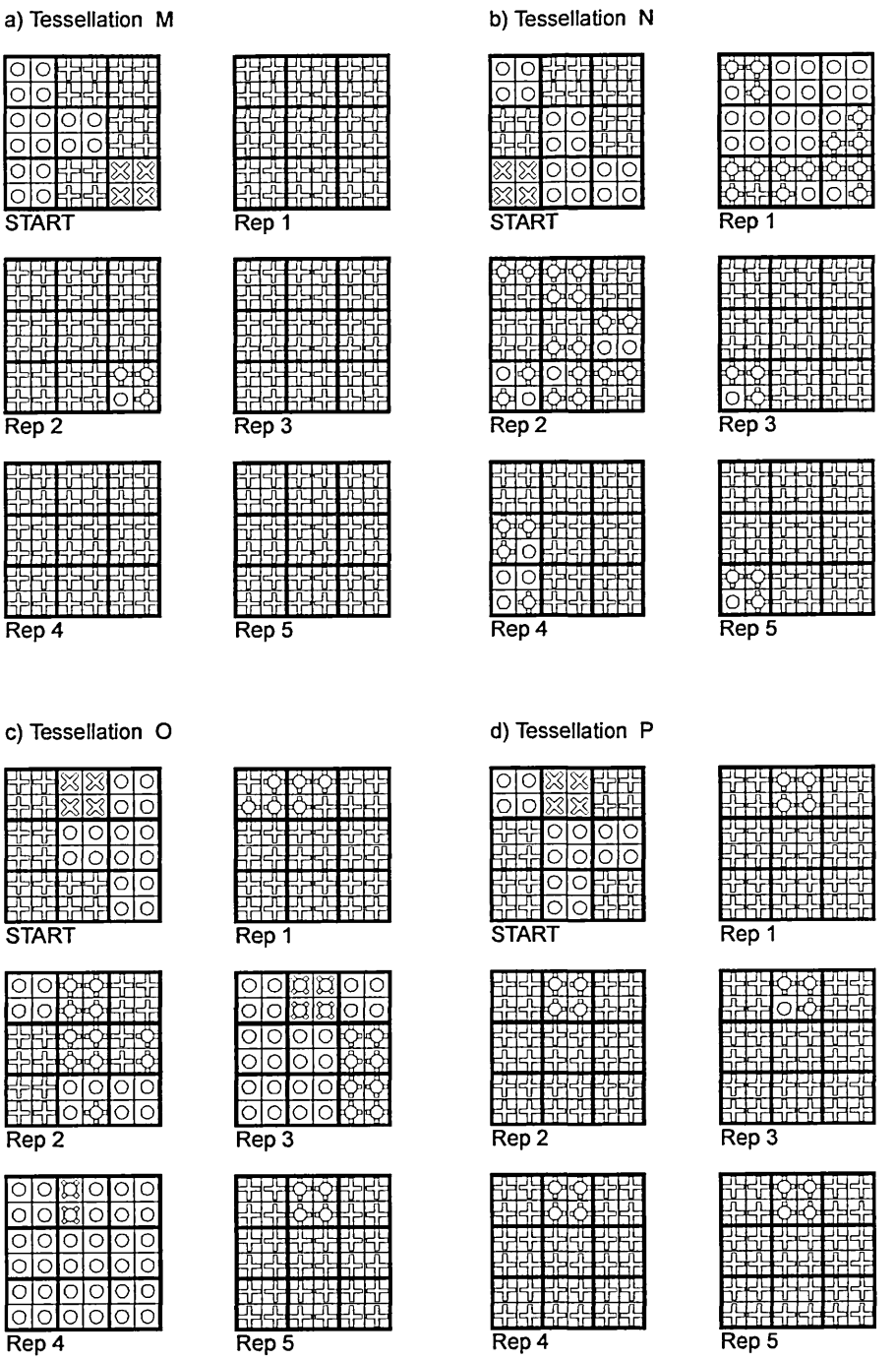


Figure 5.4. Example maps showing spatial distribution of species in the 3 x 3 tessellated agar tile arrays (1:4:4 inoculation ratio). See page 103 for full details.

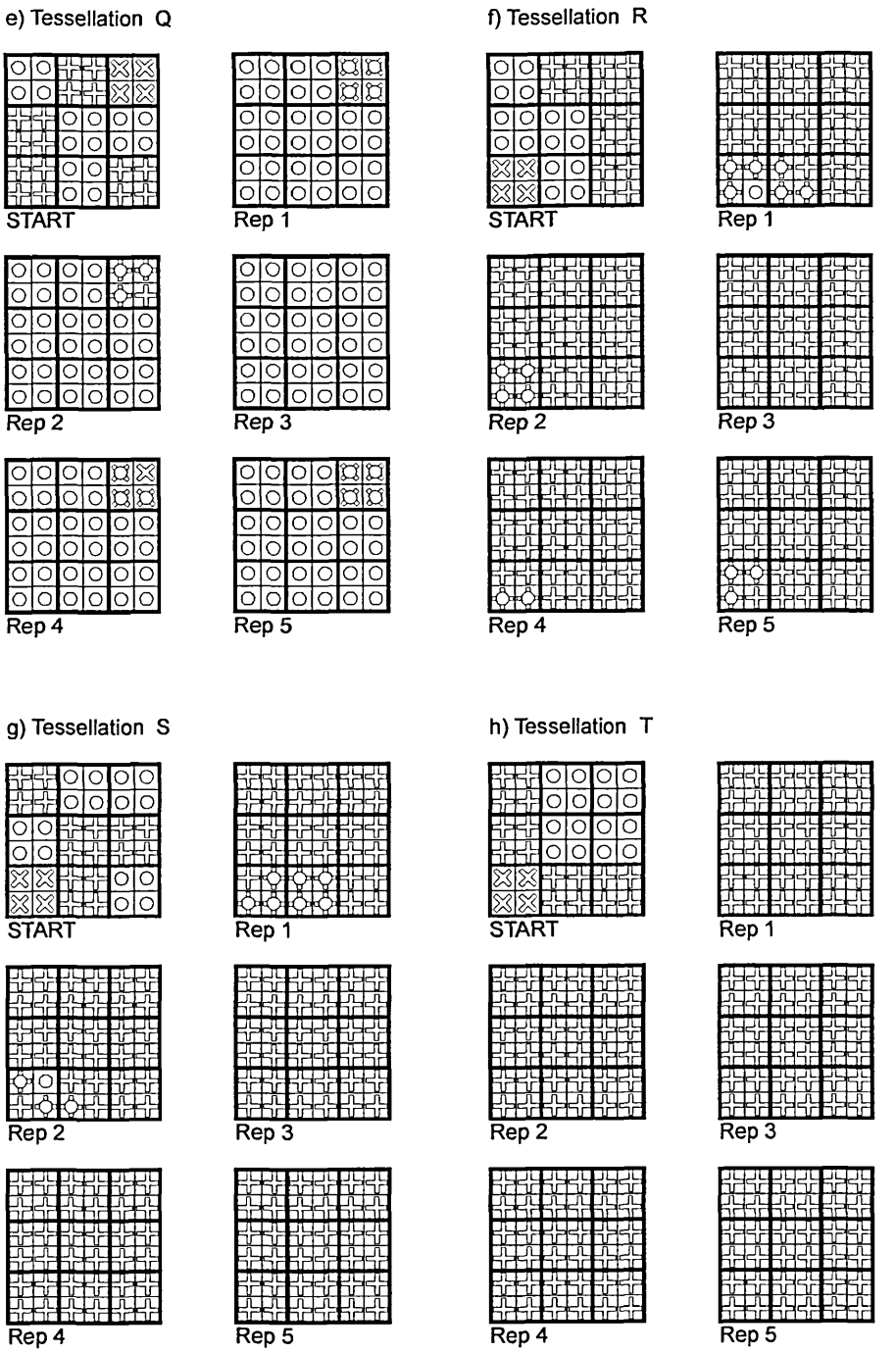


Figure 5.4. Example maps showing spatial distribution of species in the 3 x 3 tessellated agar tile arrays (1:4:4 inoculation ratio). See page 103 for full details.

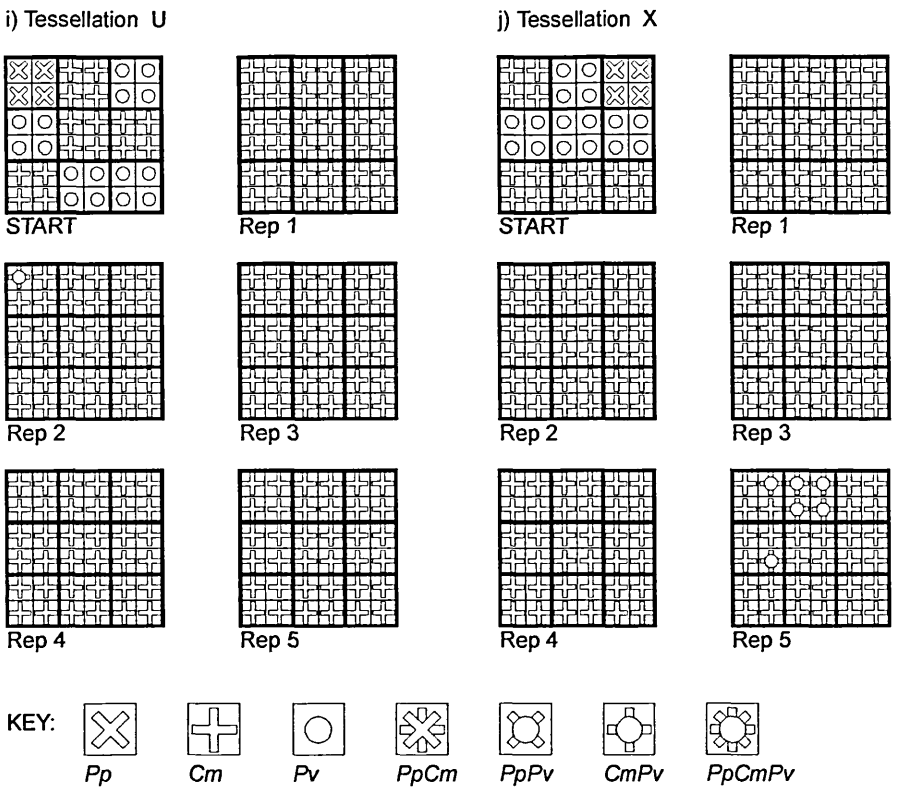


Figure 5.4. Example maps showing spatial distribution of species in the 3 x 3 tessellated agar tile arrays (1:4:4 inoculation ratio) at the onset of the experiment and after 7 weeks incubation. Bold lines denote air-gaps between tiles, fine lines denote quadrants, which were sampled, and species occupancy determined (see text). (a) Tessellation M; (b) Tessellation N; (c) Tessellation O; (d) Tessellation P; (e) Tessellation Q; (f) Tessellation R; (g) Tessellation S; (h) Tessellation T; (i) Tessellation U; (j) Tessellation X. Dimension of the tile is 1 cm^2. Symbols indicate the species isolated from each quadrant.

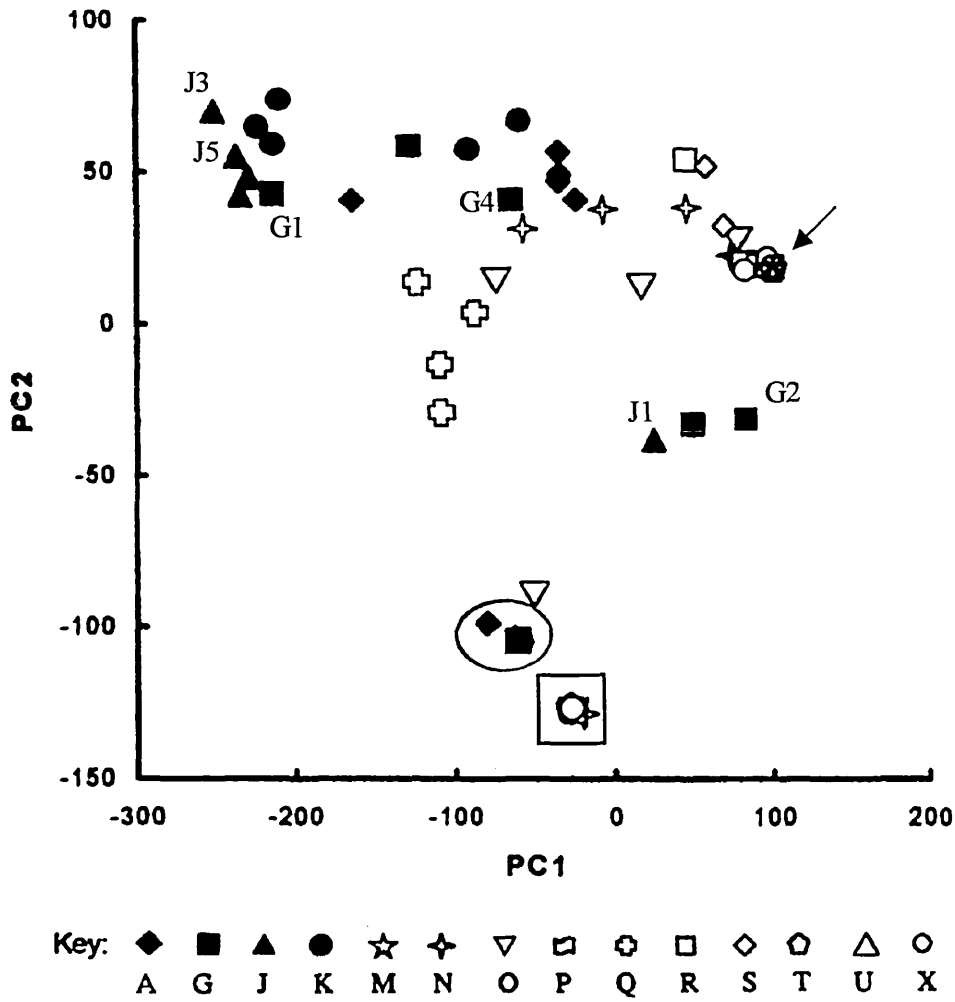


Figure 5.5. Plot of first and second principle components from analysis of interface classes and state transition classes of tessellated agar tile arrays. Open symbols denote 3 x 3 arrays, closed symbols denote 6 x 6 arrays; squared cluster denotes start configuration for 3 x 3 arrays; circled cluster denotes 6 x 6 arrays. Symbols adjacent to J1, J3, J5, G1, G2 and G4 indicate the PCA positions of the tessellations presented in Figure 5.6. Arrow indicates the clustering of 20 coincident data points for the 3 x 3 arrays.

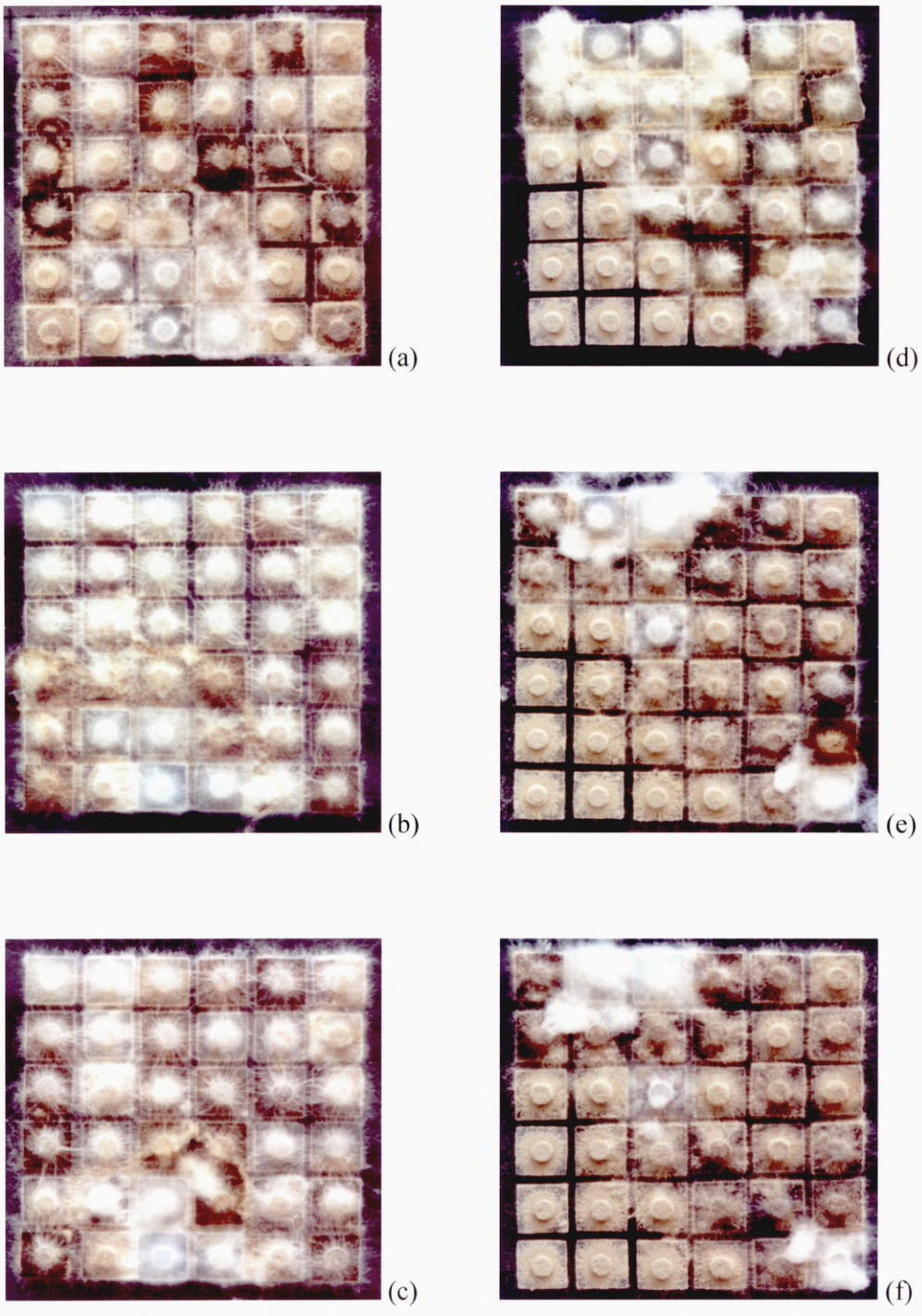


Figure 5.6. Example photographs of 6 x 6 grid, three species tile interactions after 7 weeks incubation at ambient temperature. Tessellation G (a) replicate 1, (b) replicate 2, and (c) replicate 4; and Tessellation H (d) replicate 1, (e) replicate 2, and (f) replicate 4.

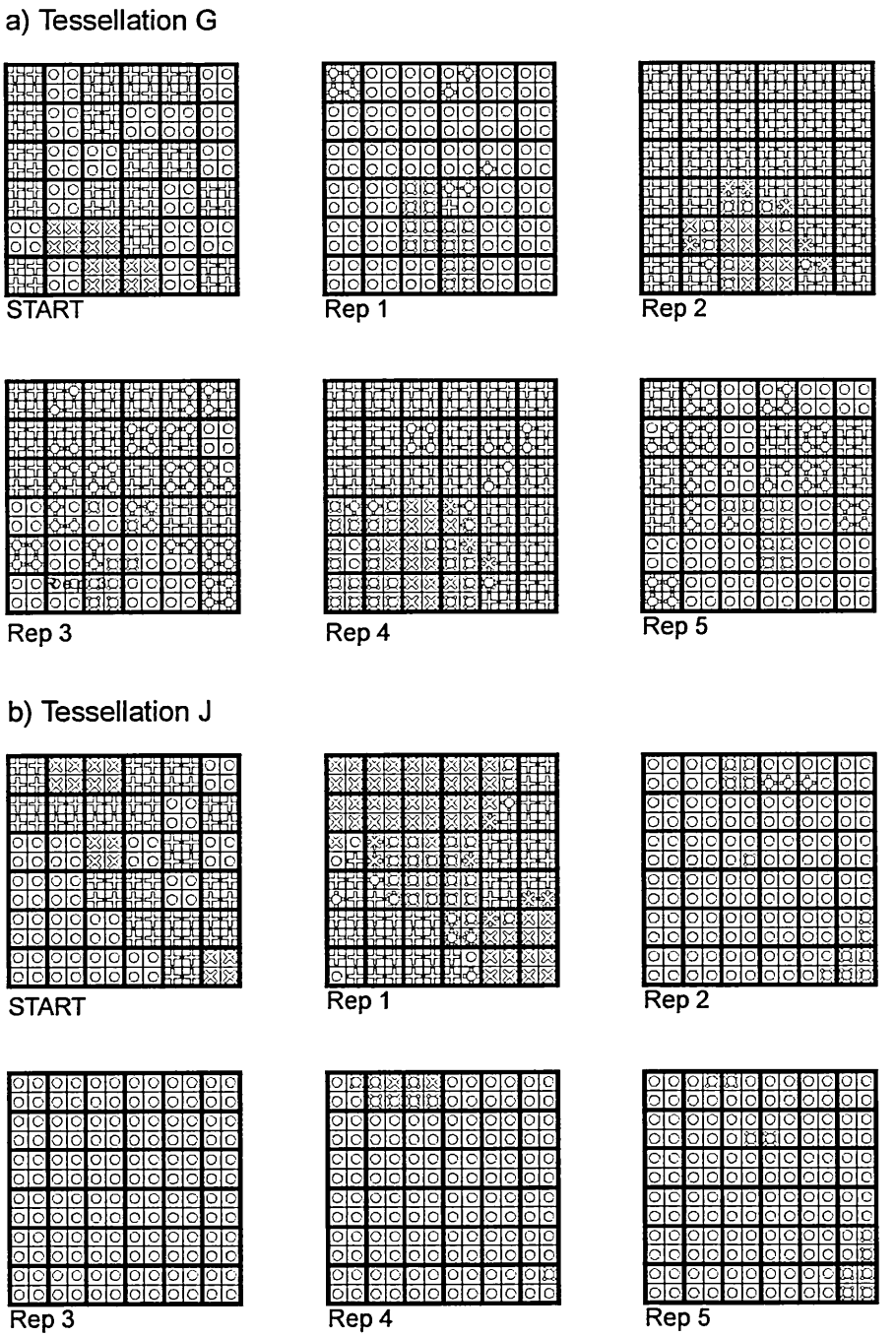


Figure 5.7. Example maps showing spatial distribution of species in the 6 x 6 tessellated agar tile arrays (1:4:4 inoculation ratio) at the onset of the experiment (START) and after 7 weeks incubation. (a) Tessellation G; (b) Tessellation J. Tile dimensions and symbols as in Fig. 5.4.

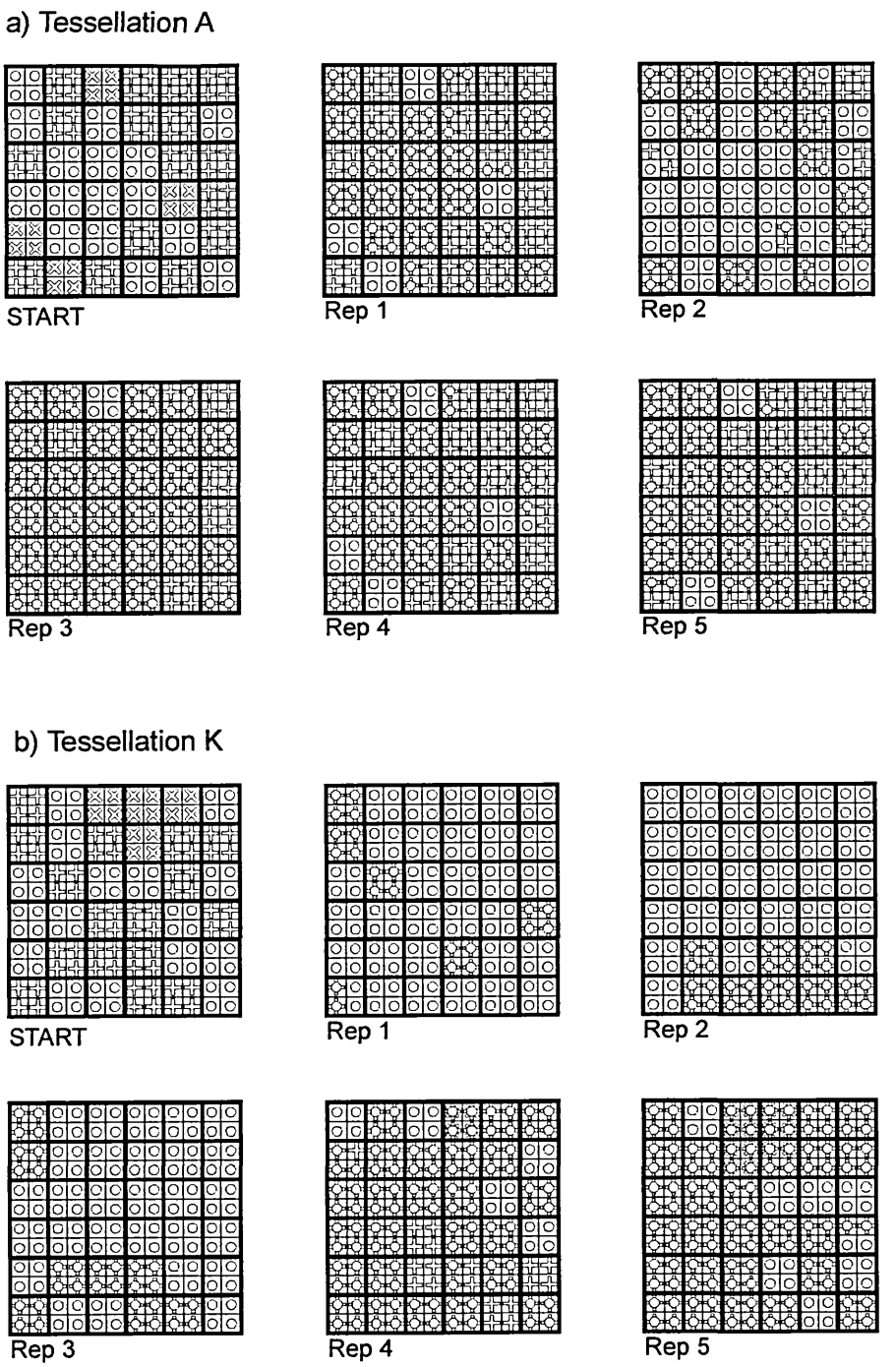


Figure 5.8. Example maps showing spatial distribution of species in the 6 x 6 tessellated agar tile arrays (1:4:4 inoculation ratio) at the onset of the experiment (START) and after 28 weeks incubation. (a) Tessellation A; (b) Tessellation K. Tile dimensions and symbols as shown in Fig. 5.4.

effective dispersal, early arrival and early exit as combative or stressful conditions intensify (Rayner & Boddy, 1988).

This explanation highlights a potential limitation of the method used to assess interaction outcome or more precisely the spatial distribution of species at a given time point. The presence of *P. variotii's* spores within a particular tile could result in false positive occurrence of *P. variotii* even if no actual viable mycelium was present during the assessment of interaction outcome.

Mapping complex spatial and temporal data for analysis has often proved problematic in studies involving heterogeneity as expressing and quantifying heterogeneity is difficult (White *et al.*, 1998). However, the approach taken in these studies show that both spatial (classification of interface classes) and temporal aspects (state transitions) of community development may be represented for numerical analysis. Indeed, the non-conventional use of principle components analysis (PCA) to statistically describe key spatial and temporal features of the tessellations proved to be highly effective method of summarising and visualising the complex species distribution maps produced. This approach facilitated a more rigorous comparison of the interaction outcome data than by subjective visual analysis.

PCA was particularly useful to show that the interaction outcomes of replicates for the 3 x 3 and 6 x 6 tessellations appeared to be neither random nor fully deterministic, as is demonstrated by the clustering and scattering of individual data points. The stochasticity displayed in the outcomes is an interesting feature and can often be found in such multi-component systems. Theory suggests that the influence of stochasticity

on interaction outcome is affected by the spatial scale of the system, with larger systems tending to behave more consistently due to the averaging out of stochasticity. Indeed, Horsthemke & Lefever (1984) confirmed this theory by a general result applicable to any system subject to internal (microscopic) fluctuations, i.e. the magnitude of the fluctuations in macroscopic quantities (e.g. relative proportion of species) is inversely proportional to the volume of the whole system. If this theory were applied to our experimental system it would predict that the outcomes of the large-scale tessellations should be more consistent between replicates compared to a smaller scales. However, such behaviour was not apparent in these experiments. For example, after 7 weeks incubation the replicates of the 3 x 3 tessellations gave more consistent outcomes compared to the large scale 6 x 6 tessellations with 20 out of 50 coincident data points and a further 18 proximal to these points observed during the 3 x 3 interactions. A plausible explanation for these observations is that the spatial scale affects the duration of transient behaviour and therefore smaller scale experiments achieve a more stable outcome faster than the larger tessellations. This feature of the interaction dynamics could therefore be considered to be associated with the time taken for an individual species to cross from one side of the microcosm to the other. Therefore, because the crossing times in larger systems are greater the dynamic timescale will be longer.

The low occurrence of *P. placenta*[1] in the multi-species interactions may be related to its low initial presence at time of inoculation. The higher inoculum of *C. marmorata* may have increased its combative and/or defensive capacity against *P. placenta*[1] leading to the replacement of the latter species. Indeed, this hypothesis is supported by work of Holmer & Stenlid (1993) who found that inoculum size was of importance in

determining the outcome of fungal interactions in wood. For example, Holmer & Stenlid (1993) found that the weakest competitor in their experiment, *Coniophora* sp., was capable of replacing *Heterobasidion annosum* when the initial inoculum size of the former was above 75%. The outcome of the interaction was the reverse when the initial inoculum size was below this level (i.e. *H. annosum* replaced *Coniophora* sp.). The effect of inoculum size therefore suggests that larger mycelial domains may have a relatively higher success in competitive confrontations. To account for this feature, further multi-species interactions, described in 5.3, were carried out using equal proportions of inocula, and specifically investigate the effect of spatial arrangement on interaction outcome.

5.3. SMALL-SCALE (3 x 3) THREE SPECIES INTERACTIONS (EQUAL INOCULUM PROPORTION)

5.3.1. Materials and methods

Simultaneously inoculated binary (2 x 1) tile interactions were constructed as described in section 2.4. Eighteen replicates for each interaction were prepared and incubated in the dark at a constant temperature of 15 °C. Six replicates of each tessellation were harvested after 1, 3 and 5 weeks and their interaction outcomes were assessed. The outcome interaction data were used to form a hierarchy of combative ability to predict the community development of the multi-species 3 x 3 interactions.

For the 3 x 3 tessellations, simultaneously inoculated tiles of *P. placenta*[1], *C. marmorata, and P. variotii* were confronted in 3 different starting configurations

(labelled A, B, C) all in the ratio 1: 1: 1 respectively. The starting layouts for these tessellations are shown in Figure 5.8. These configurations were chosen because they display heterogeneous species distribution, and that no adjacent tile was of the same species. Eighteen replicates of each tessellation were constructed and incubated in the dark at a constant temperature of 15 °C. Six replicates of each tessellation were harvested after 1, 3 and 5 weeks and their interaction outcomes were assessed. The variation between replicates for each tessellation was analysed using Levene's test (Levene, 1960).

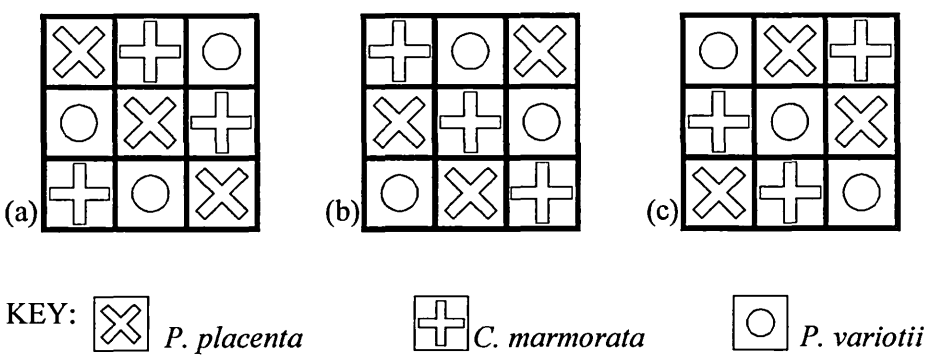


Figure 5.9. Example maps showing the spatial distribution of fungal species in the 3 x 3 tessellated agar tile arrays (equal proportion) at the onset of the experiment. Bold lines denote air-gaps between individual tiles. Dimension of each tile is 1 cm^2. Symbols indicate the species isolated from each quadrant.

5.3.2. Results

The outcomes of the 2 x 1 binary tile interactions indicated that *C. marmorata* defended its territory against *P. placenta*[1], whilst often invading *P. placenta*[1] *'s* domain

but no replacement occurred. *P. placenta*^{1} usually defended its territory against *P. variotii* and *vice versa*. *P. variotii* was invaded by and subsequently replaced by *C. marmorata*, whilst *C. marmorata* defended its territory against *P. variotii* (data shown in Table 3.8).

The interaction outcomes of the 3 x 3 tessellated agar tile interactions are expressed as species spatial distribution maps and are presented in Figures 5.10. - 5.12. Visual analysis of these results indicated that the temporal development of the species present in tessellation A were shown to be relatively consistent between replicates during the first 3 weeks with *C. marmorata* replacing *P. variotii* tiles. Subsequently, *C. marmorata* and *P. placenta*^{1} produced variable interaction outcomes depending on replicate, which resulted in deadlock, replacement of *P. placenta*^{1} by *C. marmorata* or the replacement of *C. marmorata* by *P. placenta*^{1}. *P. variotii* was also shown to emerge from a few tiles at this final sampling time point.

In tessellation B, an initial replacement of *P. variotii* by *C. marmorata* was displayed. However, at 3 weeks incubation the *P. variotii* tile in the lower left-hand-side adjacent to a diagonal of *P. placenta*^{1} colonised tiles, showed no presence of *C. marmorata* until 5 weeks at which time the *P. variotii* tile was colonised by *C. marmorata.* The final outcome after five weeks showed a deadlock interaction between *P. placenta*^{1} and *C. marmorata.*

Tessellation C displayed the most heterogeneous interaction outcomes between replicates. In general, *C. marmorata* appeared to replace both *P. variotii* and *P.*

placenta[1] domains to varying extents over the 5 week period, thus resulting in the variable interaction outcomes displayed.

Plots of the 1st and 2nd principle components, presented in Figure 5.13, displayed clustering and scattering of data that strongly agreed with the visual patterns of interaction outcome shown in all tessellations. Generally, they indicate that for tessellations A and B, interaction outcomes between replicates become more similar as incubation proceeded. Interaction outcomes remained variable in tessellation C during the 5 week period. Levene's test showed that the standard deviation of the number of tiles occupied by *P. placenta*[1] and *C. marmorata* were consistent across the initial arrangements for harvests weeks 1 and 3. However, at the week 5 sampling, the number of tiles occupied by *P. variotii* were higher for tessellation C than A (Mann-Whitney test $W = 22$; $p = 0.0074$) and varied more (Levene's test statistic = 9.474; $p = 0.012$). However, no tiles were occupied at this time on tessellations B.

5.3.3. Discussion

The initial spatial configurations of these tessellations attempt to limit the formation of lateral aggregates of individuals, and hence any potential effects of crossing times resulting from patches of species. The aim of such an approach was to allow the investigation of nearest neighbour effects on the interaction dynamics within a complex large-scale community. However, in Tessellation B (Figure 5.7b) the lower left-hand corner tile occupied by *P. variotii* was replaced by *C. marmorata*, despite the lack of a lateral *C. marmorata* nearest neighbour, or replacement of the nearest neighbour *P. placenta*[1] first to *C. marmorata* and then subsequent replacement of the

P. variotii occupied tile. This result suggests the influence of diagonal nearest neighbour tiles in the development of the community. Significantly, the diagonal aggregate of *P. variotii* in Tessellation C, appears to be related to the persistence of this species over time.

The intransistive hierarchy of combative ability predicted from the binary tile interaction data (see 3.3.5) suggests that *C. marmorata* would rapidly replace *P. variotii*, and then coexist with *P. placenta*[1], whilst defending its own spatial domain. However, comparision of this prediction to the community development shown in the 3 x 3 interactions suggests that this prediction is only valid for the development shown in tessellation B. Analysis of the species spatial distribution maps demonstrate the survival of *P. variotii* after 3 (Tessellation B) and 5 (Tessellation C) weeks, compared to the replacement of *P. variotii* by *C. marmorata* after 3 weeks in the 2 x 1 confrontations. Furthermore, within Tessellations A and C (which display maximum partitioning of *C. marmorata*) some prescribed *C. marmorata* tiles became colonised by, or replaced by *P. placenta*[1] (arrows in figure 5.9). These data, in combination with those described in 5.2, indicate that hierarcicial rules, relating to combative ability, derivied from pairwise interactions may not be sufficient to predict the overall outcomes of these larger-scale multi-species interactions. Behaviour of two fungal individuals is particular to that combination under the prevailing incubation regime. Therefore, introduction of additional species will influence the interactive functioning of the system, the operation of which cannot be predicted by measures indicating an organism's behaviour during interactions with individual fungi in isolation (Sturrock, *et al.*, 2002). Similar inferences have also been reported previously (Stahl & Christensen, 1992; White, *et al.*, 1998).

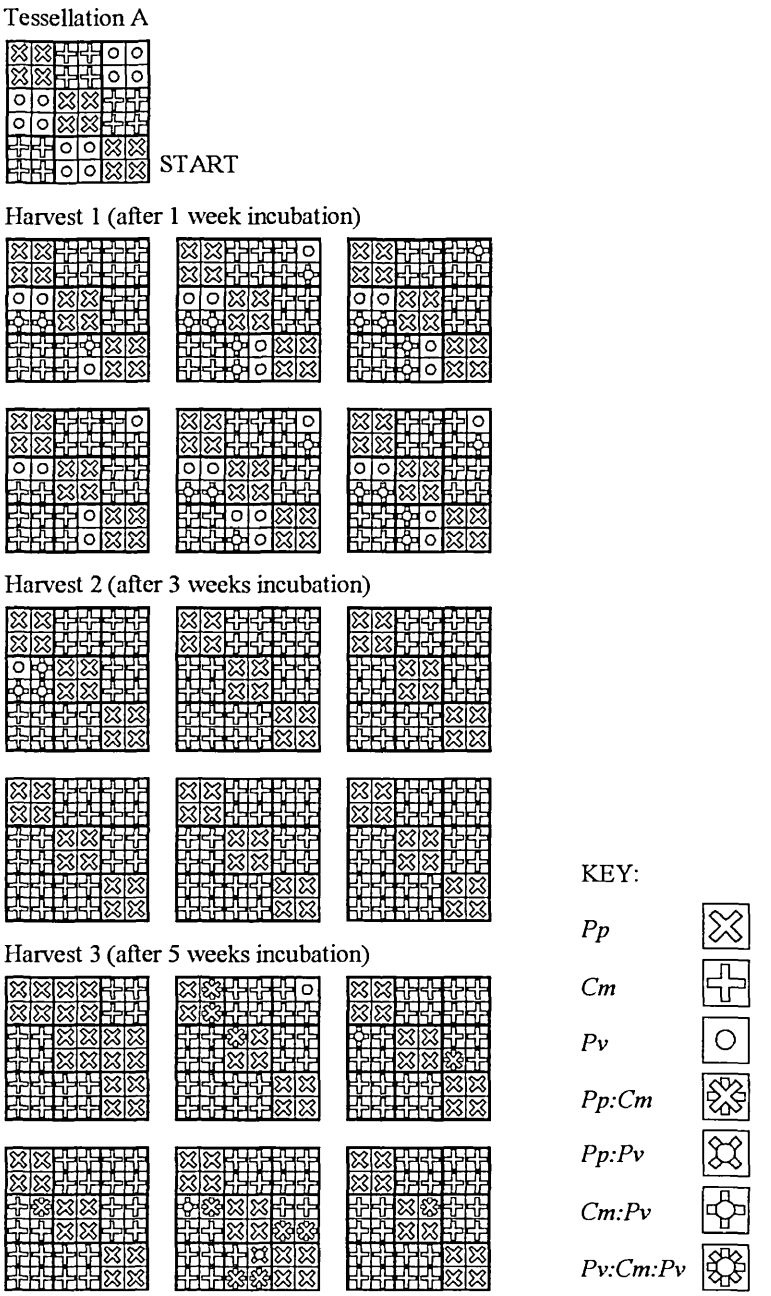


Figure 5.10. Spatial distribution maps of fungal species in the 3 x 3 tessellated agar tile array 'A' at start of interaction and then after 1, 3, 5 weeks incubation. Bold lines denote air gaps between individual tiles, fine lines denote quadrants that were plated to determine interaction outcome.

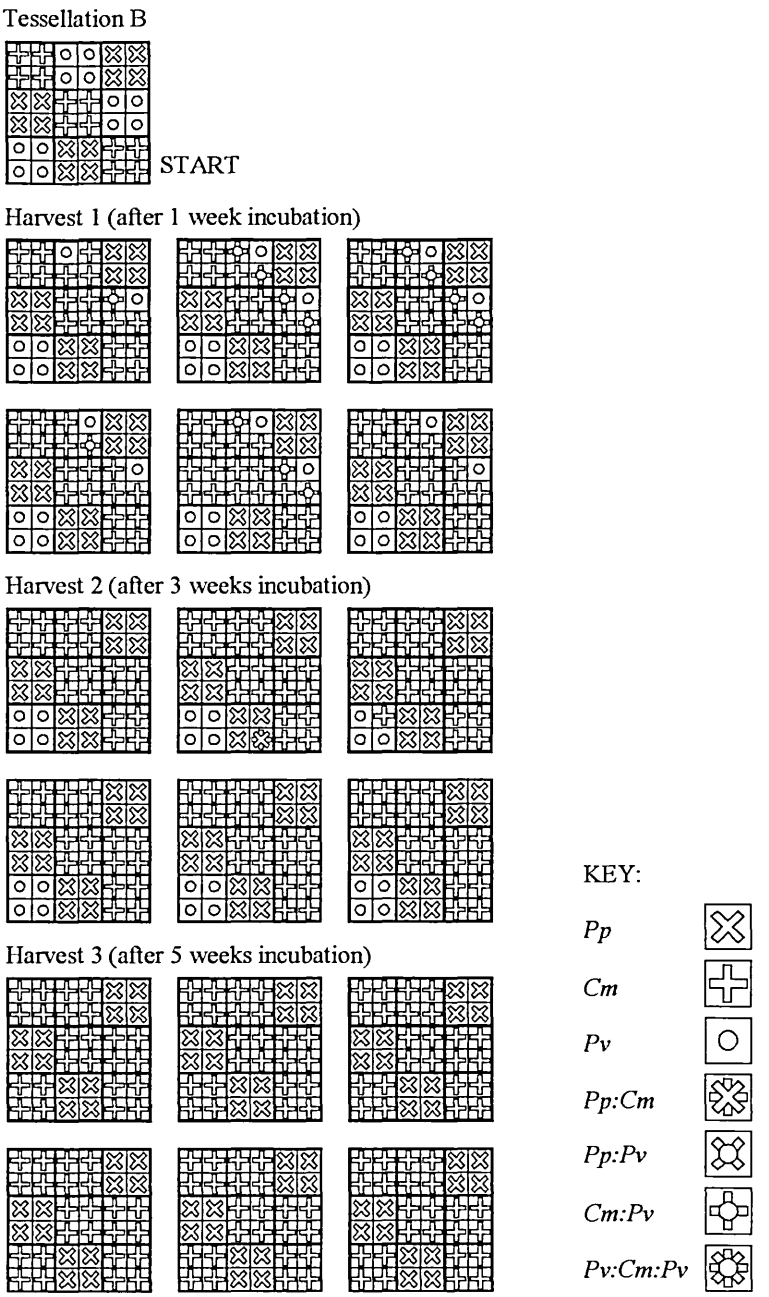


Figure 5.11. Spatial distribution maps of fungal species in the 3 x 3 tessellated agar tile array 'B' at start of interaction and then after 1, 3, 5 weeks incubation. Bold lines denote air gaps between individual tiles, fine lines denote quadrants that were plated to determine interaction outcome.

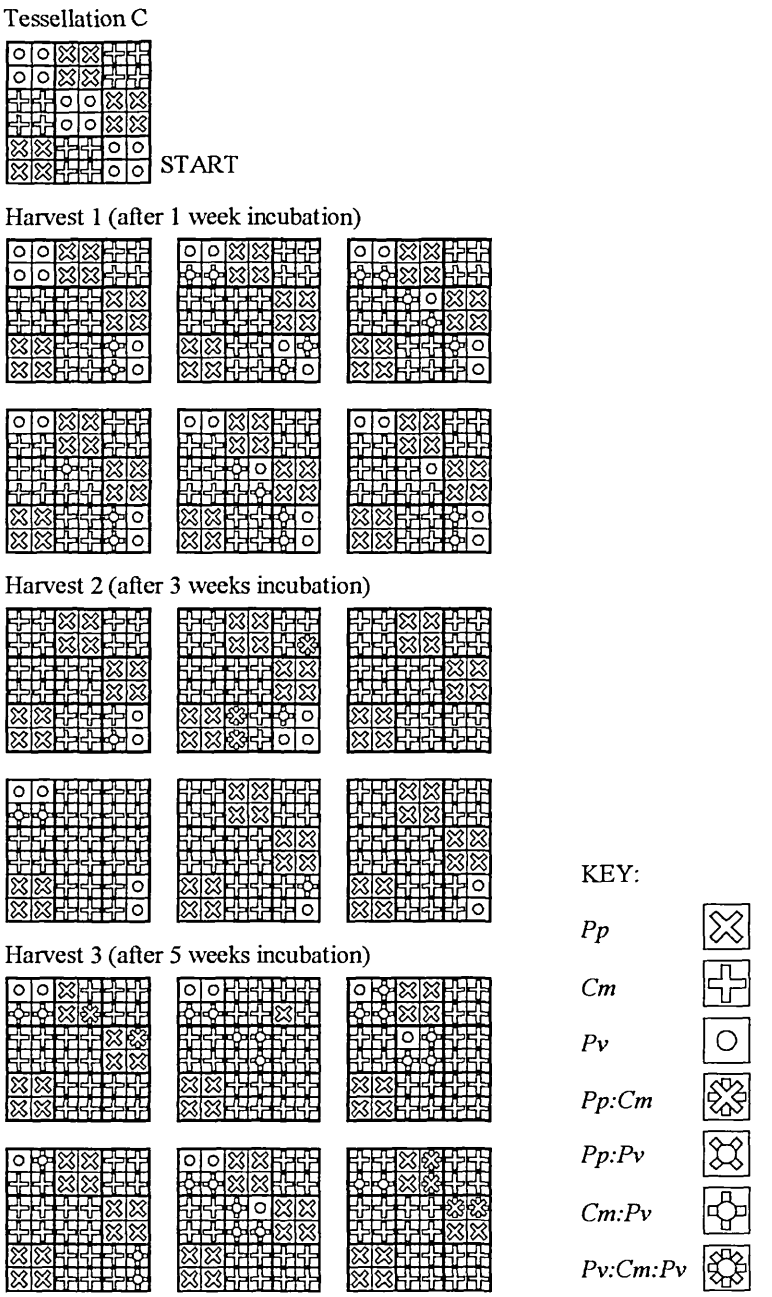


Figure 5.12. Spatial distribution maps of fungal species in the 3 x 3 tessellated agar tile array 'C' at start of interaction and then after 1, 3, 5 weeks incubation. Bold lines denote air gaps between individual tiles, fine lines denote quadrants that were plated to determine interaction outcome.

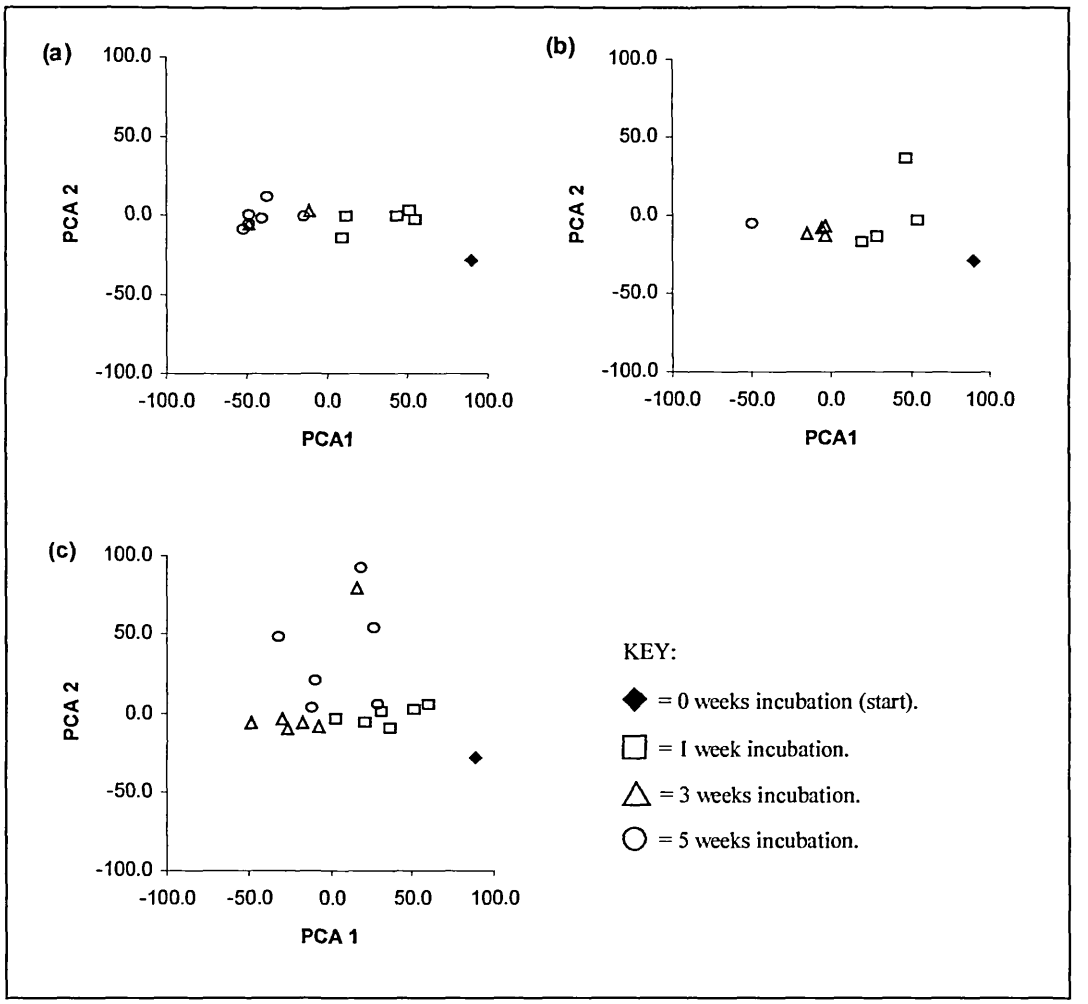


Figure 5.13. Plots of first and second principal components from analysis of interface classes and state transition classes of 3 x 3 equal proportion tessellated agar tile (a) Tessellation A (b) Tessellation B, and (c) Tessellation C. Symbols indicate incubation at 15 °C after 0, 1, 3, and 5 weeks.

The results show that the amount of heterogeneity displayed among replicates of tessellations is a consequence of the number of species present, irrespective of tessellation arrangement scale. Spatial distribution maps and the PCA of the development for the three species tessellations indicate the effects of time and spatial

arrangement on outcome variability (Figure 5.12). Variation in outcome between replicates of the same initial spatial arrangement suggests that the outcome may be sensitive to differences in starting condition below detectable limits in these experiments. However, it was apparent that replicate heterogeity of tessellations A and B appeared to converge over time. These data, if extrapolated to the natural environment, suggest that the effect of enviromental stochasticity on interaction outcome may become less significant over prolonged periods. For example, fungal succession on leaf litter of a single species collected from a single source, but then allowed to decompose in different habitats, produced a similar sequence of events but within different timescales (Frankland, 1998). In contrast, for tessellation C, replicate heterogeniety developed and presisted during the experiement. It is therefore not possible to make general observations on the effect of environmental stochasticity on interaction outcome.

Results of these three species interactions demonstrate that species survival (akin to diversity in this system) is dependent on the initial spatial arrangement of those species. Spatial pattern is an essential factor controlling species dynamics and different spatial arrangements produce different degrees of variability among replicates. The persistence of *P. variotii* in Tessellation C, and the colonisation of *C. marmorata* tiles in Tessellation A & C, could be considered as experimental evidence supporting concepts of stable coexistence of metapopulations due to appropriate spatial distribution or habitat subdivision (e.g. Robinson, *et al.*, 1993). Such interactions have been theoretically demonstrated in a number of systems (Tilman, 1994), and habitat patchiness is generally considered to promote biodiversity (e.g. Halley, *et al.*, 1996). Although patchy environments may encourage a diversity of function, as observed

here, generalisation of this is problematic, since species' function may overlap to a sufficient extent so as to collapse any partitioning from spatial patterning.

5.4. CONCLUSIONS

The experiments described in this Chapter have attempted to study the interspecific interaction of three fungal species in both small- (3 x 3) and large-scale (6 x 6) multi-species interaction arenas. The results demonstrate that the uses of combative hierarchy information based on data derived from the outcome of pairwise confrontations are unsuitable for the prediction of multispecies fungal interactions. The most plausible explanation for this is because the behaviour of two individuals is likely to be particular for that combination. Therefore, the introduction of a third species will influence the interactive behaviour of the system, the operation of which is not possible to predict from the behaviour of one species with another in isolation. In general, this type of effect has been termed 'interaction modification' and refers to situations where the outcome of interaction between two species is altered by the presence of a third (Billick & Case, 1994, cited in Boddy, 2000). Furthermore, the results also show that both inoculum size and spatial distribution of species influence the development of the multi-species interactions described in this Chapter, the effects of which are not possible to determine from pairwise interaction studies. These results therefore challenge the validity of combative hierarchy rules, derived from pairwise confrontations, used in the prediction of fungal community development not only in *in-vitro* but also perhaps in the natural environment. However, as Boddy (2000) highlighted, more research in the area of three- or higher-way interactions is required to understand the pattern of development in natural fungal communities.

CHAPTER 6. OCCURRENCE OF A MORPHOLOGICAL VARIANT OF *P. PLACENTA*

6.1. INTRODUCTION

During small- and large-scale confrontations between *P. placenta* and *C. marmorata* (described in 3.3.5 and 4.2), visual observations of the interaction indicated that the morphological features of *P. placenta* were different to those observed during the preliminary binary tile interactions described in 3.3.1 and 3.3.2.

A possible explanation for the occurrence of this morphological variation of *P. placenta* could be the contamination of the original stock culture with a closely related strain of *P. placenta.* Indeed, White *et al.* (1996) described the isolation of several distinct strains of *P. placenta* during investigation of the fungal community structure in the keelson and sister keelson of the Frigate Unicorn. Therefore, it could be possible that an original stock slope may have contained a mixture of two (or possibly more) closely related strains of *P. placenta* that display different morphological characteristics, and during routine subculture a variant form was accidentally isolated.

Another possible explanation for occurrence of the morphological variation could be due to prolonged subculture and storage in artificial culture media. For example, many species of *Fusarium* tend to be highly variable in morphology in culture, as nutrient concentrations are often considerably higher than in their natural habitat (i.e. often very dilute solutions in the water conducting vessels of plants). Hence there is intense selection favouring any

variant capable of growth in the unnatural condition of artificial culture (Carlile & Watkinson, 1994). Another example is that subculture by means of cores of agar bearing mycelium may often lose the ability to sporulate as there is no selection against mutants defective in sporulation (Carlile & Watkinson, 1994). In relation to *P. placenta,* it is proposed that continued subculture in artificial media could have affected certain metabolic pathways associated with degradation of its natural resource, wood. The degradation of wood by decay fungi involves a complex range of enzymatic and non-enzymatic metabolic processes (Rayner & Boddy, 1988) therefore, growth in a homogeneous artificial laboratory medium could influence the activity of these metabolic processes and perhaps others relating possibly to combative ability.

To investigate these possible explanations the experiments described in this Chapter aims to:

- Isolate pure cultures of each *P. placenta* variant and compare their morphological characteristics.
- Investigate the effect of culturing the *P. placenta* variant on natural substrata (e.g. wood) in an attempt to 'revert' the species to its original form.
- Compare the combative ability of each *P. placenta* variant against *C. marmorata.*

6.2. MATERIALS AND METHODS

All materials and methods used in this Chapter are described in section 2.5.

6.3. RESULTS AND DISCUSSION

6.3.1. Morphological characteristics and growth rates

Pure cultures of *P. placenta*[1] and *P. placenta*[2] were produced using the hyphal tipping method described in section 2.5.3. All *P. placenta*[1] and *P. placenta*[2] cultures produced were shown to display the same macro-morphological features as their respective original stock plates (i.e. *P. placenta*[1] grew as white cottony mycelium and *P. placenta*[2] grew as grey-white appressed, submerged mycelium. See Figure 6.1). Differences in growth rate were also apparent between the two growth forms of *P. placenta*. *P. placenta*[1] displayed a slightly higher rate of mycelial extension compared to *P. placenta*[2] when incubated at ambient temperatures (Table 6.1). However, results of the extracellular enzyme tests were the same for both *P. placenta*[1] and *P. placenta*[2].

Table 6.1. Mean growth rates, standard error of the mean and extracellular enzyme tests for *P. placenta*[1] and *P. placenta*[2] incubated at ambient temperature.

	Species	
Factor	*P. placenta*[1]	*P. placenta*[2]
Mean growth rate (mm day $^{-1}$)	14.99	12.05
Standard error of mean	SD. 0.98	SD. 0.23
Laccase	Faint positive	Faint positive
Peroxidase	Negative	Negative
Tyrosinase	Negative	Negative

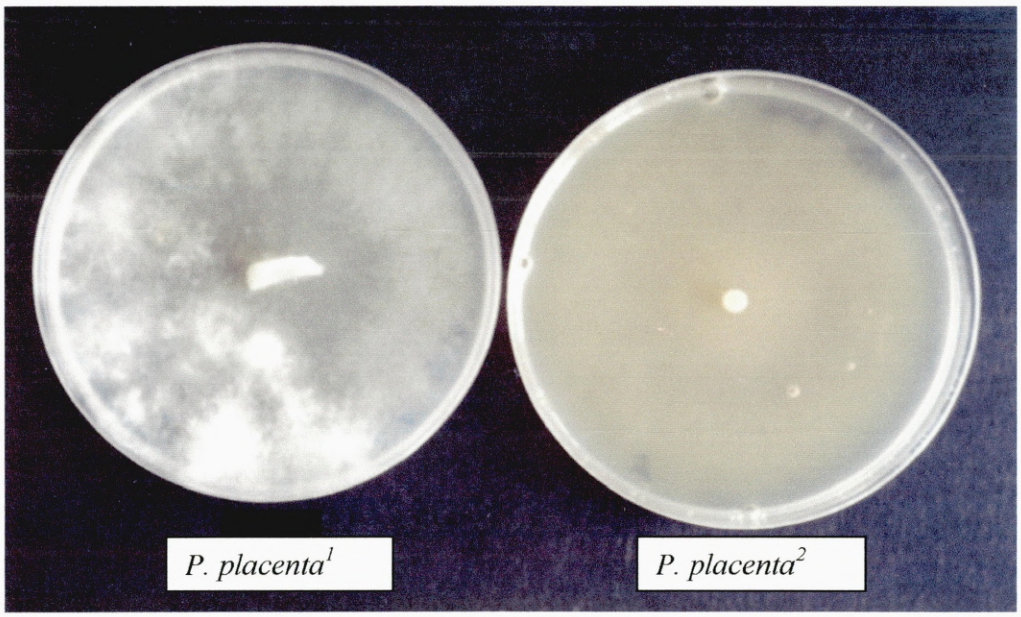


Figure 6.1. Photograph of (a) *P. placenta*[1] and (b) *P. placenta*[2] grown on 2% MEA.

6.3.2. Growth of P. placenta[2] *on wood*

The growth of *P. placenta*[2] on wooden blocks and subsequent re-isolation to agar culture resulted in the *P. placenta* isolate denoted *P. placenta*[3]. The combative ability of this isolate and the other two were subsequently compared by confrontation against *C. marmorata.*

6.3.3. Interaction studies

In general, the interactions between *P. placenta*[1] *vs C. marmorata* and *P. placenta*[3] *vs C. marmorata* displayed similar development during the interaction. Each species colonised their respective tiles after approximately 6 – 7 days and subsequently a yellow interaction zone line was formed in the air gap between the two tiles where each species mycelia came into contact. Over the following 11 weeks *C. marmorata's* tiles progressively became dark brown in colour. However, it was unclear as to what

extent either species had invaded its opponents tile and therefore assessment of interaction outcome was required using re-isolation techniques.

The interaction between *P. placenta*^{2} *vs C. marmorata* displayed a different development compared to the other *P. placenta* interactions. Initially, each species colonised their respective tiles after approximately 6 – 7 days and subsequently a yellow interaction zone line was produced at the leading edge of *P. placenta*^{2}*'s* tile. Over the following 11 weeks *C. marmorata* invaded *P. placenta*^{2}*'s* tile which progressively turned dark brown in colour. Interaction outcomes were assessed using the methods described in section 2.4.1.1 after 0, 4, 8 and 12 weeks.

The proportion of each species extant within each tile quartile after 0, 4, 8 and 12 weeks for each *P. placenta* variant are shown in Table 6.2. In general, the interaction outcome results indicated that both *P. placenta*^{1} and *P. placenta*^{3} displayed a deadlock interaction with *C. marmorata*, whereas, *P. placenta*^{2} was replaced by *C. marmorata.*

6.4. DISCUSSION

The aim of the hyphal tipping experiment was to establish whether the original P. placenta stock was contaminated with closely related strain of *P. placenta.* The results showed that all subcultures displayed the same macro-morphological features as the original stock culture suggesting that no contamination was present.

Table 6.2. Proportion of species extant during interactions between *P. placenta*[1], *P. placenta*[2] or *P. placenta*[3] against *C. marmorata* after incubation for 0, 4, 8, or 12 weeks at ambient (20 – 25 °C) temperatures, in the dark. Bold rows denote differences in interaction outcome found during interaction of *P. placenta*[2] with *C. marmorata*.

Sampling period (weeks)	Proportion of fungal species in confronted inoculated tiles					
	Tile 1			Tile 2		
***Cm* (Tile 1)**	Species			Species		
***Pp*[1] (Tile 2)**	*Pp*[1]	*Cm*	*Pp*[1] & *Cm*	*Pp*[1]	*Cm*	*Pp*[1] & *Cm*
0	0	24	0	24	0	0
4	0	22	2	24	0	0
8	0	23	1	24	0	0
12	0	22	2	24	0	0
***Cm* (Tile 1)**	Species			Species		
***Pp*[2] (Tile 2)**	*Pp*[2]	*Cm*	*Pp*[2] & *Cm*	*Pp*[2]	*Cm*	*Pp*[2] & *Cm*
0	0	24	0	24	0	0
4	0	24	0	**18**	**0**	**6**
8	0	24	0	**4**	**12**	**8**
12	0	24	0	**1**	**16**	**7**
***Cm* (Tile 1)**	Species			Species		
***Pp*[3] (Tile 2)**	*Pp*[3]	*Cm*	*Pp*[3] & *Cm*	*Pp*[3]	*Cm*	*Pp*[3] & *Cm*
0	0	24	0	24	0	0
4	0	24	0	24	0	0
8	0	22	2	24	0	0
12	0	24	0	24	0	0

Comparison of the growth characteristics of *P. placenta*^{1} and *P. placenta*^{2} were similar with only a slight reduction in growth rate and loss of aerial mycelium production for *P. placenta*^{2}. Comparison of the interaction outcomes for the three *P. placenta* isolates showed that both *P. placenta*^{1} and *P. placenta*^{3} deadlocked with *C. marmorata*, whereas, *P. placenta*^{2} was replaced. These data would suggest that through the culture of *P. placenta*^{2} on wood (to produce *P. placenta*^{3}) the altered growth form has regained its combative ability against *C. marmorata*. This finding would therefore support the theory that long-term cultivation and storage on artificial media may influence the biochemical functioning and behavioural features of the organism. Indeed, in the heterogeneous natural environment the fungal mycelium is highly dynamic, capable of switching its growth form in response to local environmental stimuli (Rayner, 1988; Rayner & Boddy, 1988). Therefore, growth in a homogeneous media, devoid of environmental stimuli could result in the organism 'switching off' non-essential biochemical pathways as a means of consolidation of the mycelial thallus.

CHAPTER 7. DEVELOPMENT OF A NON-DESTRUCTIVE ANALYSIS SYSTEM FOR THE STUDY OF INTERSPECIFIC FUNGAL INTERACTIONS

7.1. INTRODUCTION

7.1.1. General premise

A significant technical limitation of the tessellated agar tile interaction system described in previous chapters is the destructive method of analysis used to determine species occupancy within each tile. This method, involving the re-isolation of fungal species', not only requires large numbers of replicates for each tessellation but also results in the generation of data that are essentially discontinuous i.e. allowing only one sample point for each tessellation. Fungal biomass is characteristically difficult to measure quantitatively, and much of the description of fungal growth in heterogeneous environments has been based on observation (Ritz & Crawford, 1999). The application of a non-destructive analysis system could potentially allow continuous monitoring of community development and could facilitate the development of a more appropriate biological model.

7.1.2. Potential non–destructive analysis systems

Experimental consideration must be given during the development of a suitable continuous model system to the spatial resolution of data. This must be at an appropriate scale to allow for accurate mapping of community development. Also, it is important that the system does not subject the experimental microcosms to any undue stress or disturbance between sampling points. Potential systems, such as immunoblotting, (involving overlaying a nitro-cellulose membrane on the surface of

the microcosm, then detecting the presence of each species using immunological based probes) or competitive polymerase chain reaction (involving the removal of small cores from the microcosm, then identifying the presence of each species from the sample) could be used to determine the temporal occupancy of each species within the microcosms. However, such systems are still essentially destructive, subjecting the microcosms to disturbance during the course of the experiment, which could significantly influence the community dynamics of the system. In recent years, a Green Fluorescent Protein (GFP) based technology has been developed as a new vital marker for many species (see section 7.1.3. for examples). This new technology could therefore be used to label a fungal species, and in combination with computer based image analysis techniques, map the growth of the labelled organism within an *in vitro* interaction system.

7.1.3. What is GFP?

Green Fluorescent Protein is a 238 amino acid polypeptide involved in bioluminescence of aquatic invertebrates in the phylum *Cnidaria.* Interestingly, the first recorded report of such bioluminescence was by "Pliny the Elder" in the first century AD, who described the bright glow of certain jellyfish present in the Bay of Naples. However, his early development of making various objects "glow as on fire", by applying the brightly fluorescent organism, was terminated abruptly in the 79 AD with the eruption of Vesuvius (Johnson & Shimomura 1978). More recently, Chalfie *et al.* (Charfie *et al.* 1994) showed that GFP cDNA from *Aquorea victoria* could be expressed in both prokaryotic (*Escherischia coli*) and eukaryotic (*Caenorhabdtis elegans*) cells producing strong green fluorescence when excited by blue light. Although other methods are available to study gene activity and protein localisation

within the cell (e.g. β-glucuronidase (GUS), luciferase (LUC), and chloroamphenicol acetyltransferase (CAT)) such techniques are of limited use in living tissue as they all require exogenously added substrates or co-factors which are often toxic to the cells (Sheen *et al.* 1995). The only other factor apart from exposure to near UV or blue light required for GFP fluorescence is the presence of molecular oxygen (Cormack, 1998). These properties make GFP a valuable tool for the specific labelling of living cells, as no exogenous substrates are required to induce luminescence.

Within the past five years, GFP technology has been developed as a powerful and versatile analysis tool for many diverse biological processes. These include its use as a reporter of gene expression (Charfie *et al* 1994; Cormack *et al.* 1997; Dumas *et al.* 1999), a marker of subcellular protein and organelle localisation (Fernandez-Abalos *et al.* 1998; Li & Kaplan 1997; Suelmann and Fischer, 2000), and as a label to follow the development of phytopathogenic fungi within their hosts (Spellig *et al.*,1996; Vanden Wymelenberg *et al.*,1997).

7.1.4. Overview of fungal genetic transformation strategies

Genetic transformation is the introduction of foreign genes or constructions involving different parts of genes into a host species using a DNA molecule or vector. These genes, if they are successfully integrated and are operational within the host species, can then be expressed. A summary of the genetic transformation strategy for fungi is described in Figure 7.1.

A common method of transformation of fungi involves the production of protoplasts from fungal cells using various cell wall degrading enzymes. Protoplasts can then be induced to uptake the DNA molecules by chemical (normally a solution of

polyethylene glycol (PEG) and calcium chloride) or electroporation methods. Integration of foreign DNA into the host species' genome can occur through either homologous or non-homologous (ectopic) recombination events (Hooley, 1993) Examples of each of these events are shown in Figure 7.1. In this diagram homologous recombination can be seen as the replacement of allele *a* by allele *A*. In contrast, non-homologous recombination shows the integration of the entire plasmid DNA at a random site away from locus *a*. In addition to these events, multiple copies of the foreign DNA may be integrated in the form of tandem repeats (i.e. two or more copies of the DNA vector are integrated sequentially). It is theorised that this property of integration is due to the tendency of exogenous DNA's to recombine with each other rather than with the chromosome (Aleksenko & Clutterbuck, 1995). Such events have lead to the introduction of two different DNA vectors into a host species a process termed co-transformation.

To identify between transformed and un-transformed protoplasts it is necessary to use a selectable marker. Commonly, auxotrophic markers are used, but these have the disadvantage of the requirement for generating appropriate genotypes in your recipient strain (Hynes, 1996). This is often laborious and can be difficult in species without good genetic characterisation. Another method of selection is the use of drug resistance markers. Introduction of bacterial genes encoding resistance to the antibiotics bleomycin (Austin *et al.* 1990), phleomycin (Birch *et al.* 1998) and hygromycin B (Casselton & Herce, 1989; Kamper *et al.* 1995) have been found to be extremely useful selection agents in filamentous fungi.

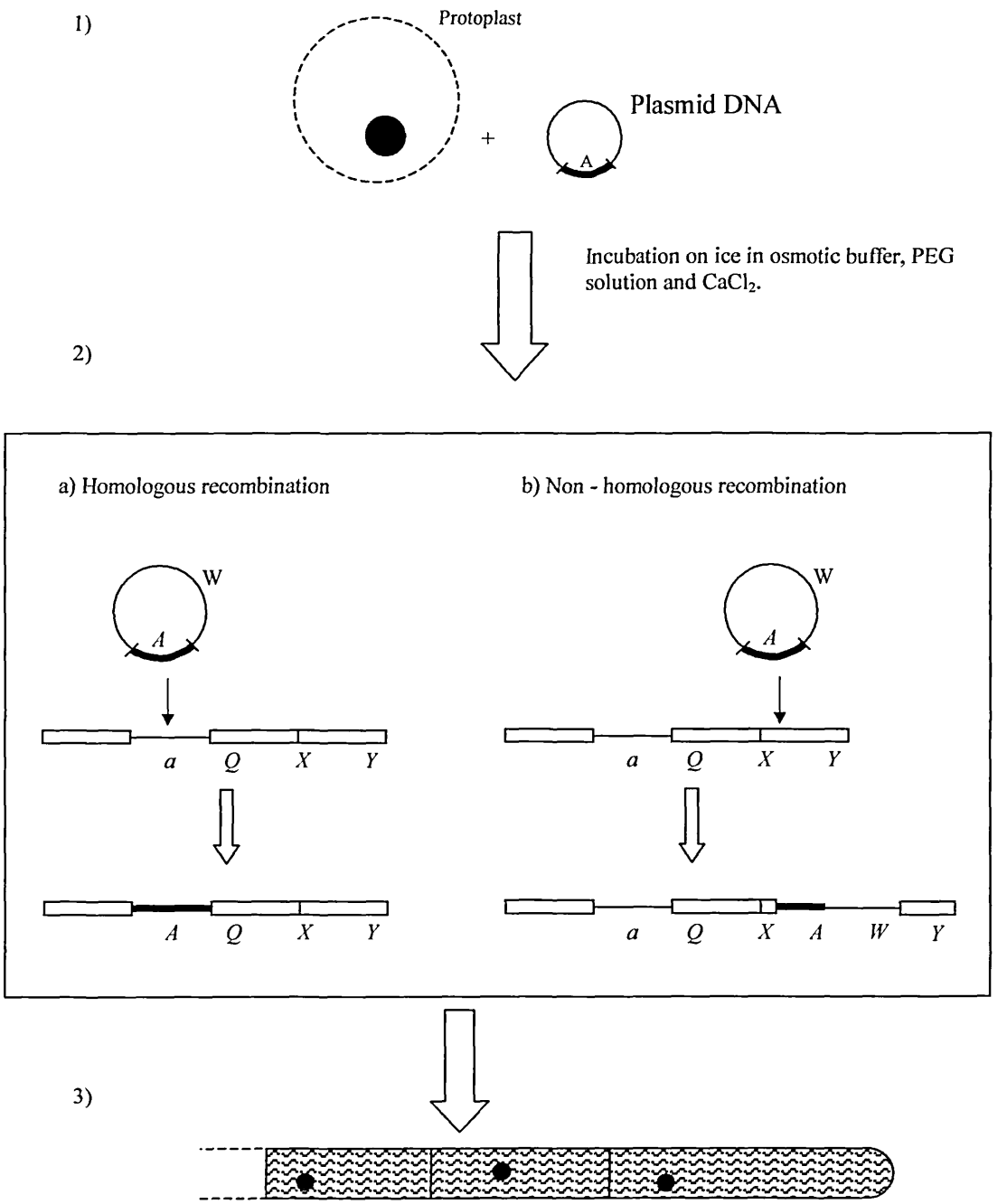


Figure 7.1. Summary of of genetic transformation in fungi. (1) Introduction of plasmid DNA to fungal protoplast. (2) Patterns of gene integration (see text for further details). (3) Return to filamentous growth with expression of foreign DNA. Diagram not to scale. See text for details.

7.1.5. Aims

The aim of this chapter was to develop a non-destructive means of mapping the spatial and temporal distribution of fungal species within an agar based tile interaction system. The methods subsequently described are part of a genetic transformation procedure to label *C. marmorata* with GFP. The transformation strategy involved the introduction of the GFP gene and a hygromycin resistance gene (used as a selection agent for successful transformation) into the genome of the host species.

7.2. MATERIALS & METHODS

7.2.1. Protoplast Production

To develop a reliable method of protoplast production from *C. marmorata* four sets of experiments were performed. In an attempt to optimise protoplast yield and regeneration each experiment investigated a different factor, namely osmotic buffer constitution, mycelia age, and exposure time to the lytic enzymes.

7.2.1.1. Preliminary protoplast production experiment

The initial protoplasting experiment was designed to investigate the effect of different osmotic stabilisers on protoplast release. Three separate studies were performed using a different stabiliser for each. These were; (*i*) 1 M sodium chloride (NaCl), (*ii*) 0.5 M magnesium sulphate ($MgSO_4$) in 50 mM maleic acid - sodium hydroxide (NaOH), (*iii*) 0.5 M mannitol in 50 mM maleic acid - NaOH. All osmotic stabilisers were adjusted to pH 5.5. Protoplast production was performed using the method described in section 2.6.2.1.

7.2.1.2. The effect of culture age and lytic enzyme exposure time on protoplast release

Protoplasts were produced using the method described in Chapter 2, section 6.2.2. Regeneration of protoplasts was assessed using the method described in section 2.6.3.

7.2.1.3. Effect of buffer pH on protoplast release and regeneration

Protoplasts were produced with 48 h old cultures using the method described in section 2.6.2.2. The effect of buffer pH was assessed at pH 5.5, 6.0, and 7.0. Regeneration was assessed using the method described in section 2.6.3.

7.2.1.4. Effect of osmotic stabiliser concentration on protoplast release.

The general method of protoplast production used is described in Chapter 2 section 6.2.2. The effect of osmotic stabiliser concentration was assessed at 0.5, 0.75, and 1 M mannitol (in 50 mM maleic-NaOH buffer pH 5.5). The initial quantity of mycelial mats used was doubled, to 8 colonies, as was the volume of the lytic enzyme digestion (14 ml). Cultures were 24 h old.

7.2.2. Assessment of C. marmorata protoplasts sensitivity to the antibiotic hygromycin B

Methods of hygromycin B sensitivity on protoplasts of *C. marmorata* are described in 2.6.2.2.

7.2.3. Fungal transformation

The genetic transformation of *C. marmorata* was attempted using 2 methods. These methods are described in section 2.7.1. and 2.7.2., respectively.

7.3. RESULTS & DISCUSSION

7.3.1. Protoplast production

7.3.1.1. Preliminary protoplast production experiment

C. marmorata protoplasts were generated from all three buffers tested, although the final yield differed for each buffer. Protoplast production was greatest with 0.5 M mannitol and lowest with 1M NaCl (table 7.1), however the overall yields were lower than that reported in previous studies using different species (Marmeisse *et al.*, 1992; Rui & Morrell, 1993; Thornewell *et al.*, 1995). Rui & Morrell (1993) found that mycelial culture age can affect production, with protoplast yield decreasing as mycelial age increased, possibly indicating senescence of older parts of the mycelium. This feature of protoplast production has been useful in the isolation of cellular components from young and old cells of filamentous fungi and in the comparison of their enzyme activities (Carlile & Watkinson, 1994).

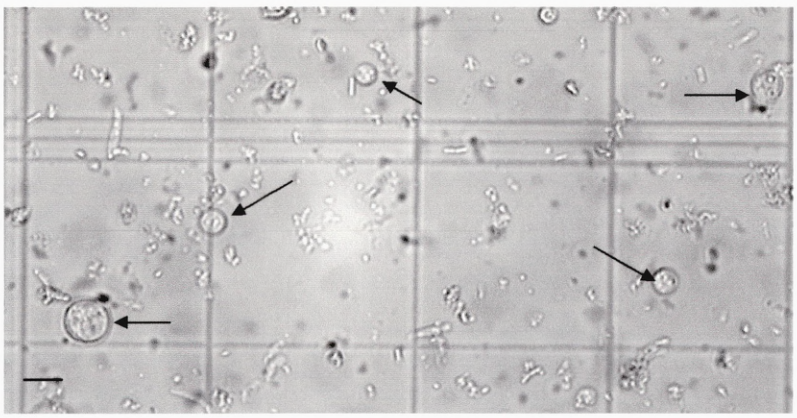
The various macro-morphological stages of protoplast regeneration are presented in Figure 7.2. Protoplast regeneration can provide a relative measure of cell viability after enzyme treatment. Protoplasts may often fail to regenerate possibly due to the lack of nuclei or due to damage at some point during or after enzyme treatment (Rui & Morrell, 1993). Regeneration of protoplasts was greatest with 0.5 M mannitol and least with 0.5 M $MgSO_4$. These findings were in agreement with those reported by Rui & Morrell (1993). A possible explanation for the poor protoplast regeneration when produced with ionic salts is that they have been found to alter the configuration and hence the activity of

enzyme molecules within the cell (Carlile & Watkinson, 1994). Mannitol, one of many sugar alcohols produced by fungi, has an important role in adaptation to natural osmotic stresses and could therefore suggest its better performance as an osmotic stabiliser.

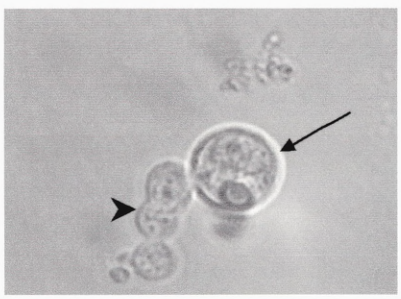
These initial investigations have shown that protoplasts can be produced from the wood decaying species *C. marmorata*. Although protoplasts yields were lower than expected optimisation of the experimental protocol is described in the following sections. Factors such as culture age, lytic enzyme exposure time, buffer pH and concentration were manipulated to improve the yield of protoplasts generated.

Table 7.1. Effect of osmotic stabiliser type on protoplast yield.

Osmotic buffer	Protoplast yield (ml^{-1})	Regeneration (%)
1M NaCl	0.4×10^6	11
0.5 M $MgSO_4$	1.2×10^6	8
0.5 M Mannitol	**3.2×10^6**	**18**

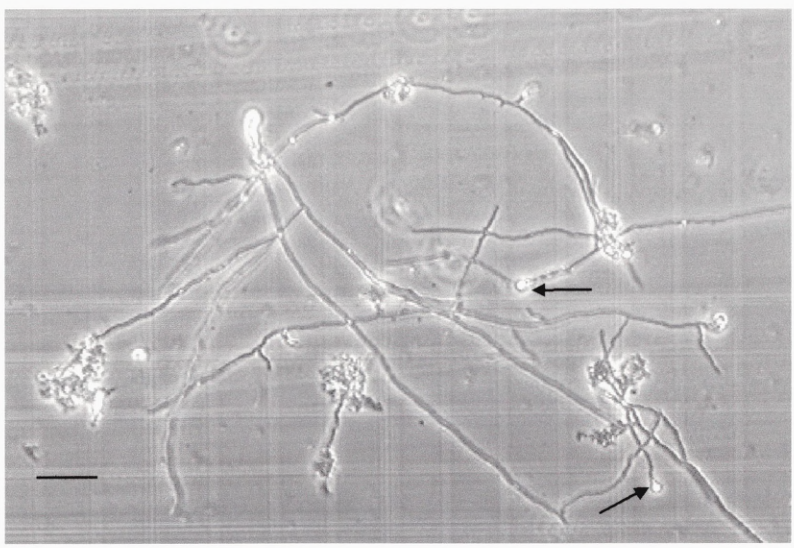


a)



b)

c)



d)

Figure 7.2. Photographs showing regeneration of *C. marmorata* protoplasts. (a) Protoplast (arrow) after 0 h incubation; bar = 10 μm, (b) Protoplast showing budding (arrow head) after 24 h, (c) Protoplast showing initial return to hyphal growth (white arrow) after 2 days, (d) Protoplasts after five 5 days incubation; bar = 50 μm. See text for details.

7.3.1.2. Effect of culture age and enzyme exposure time on protoplast release

Comparison of protoplast yields between the two culture ages indicated that 24 h cultures produced greater overall yields of protoplasts (table 7.2) using 0.5 M mannitol maleic - NaOH buffer. Protoplast yield was shown to be greatest after 30 min in the 24 h cultures, however the yield decreased after this time. The optimal enzyme exposure time for the 48 h cultures was 60 min (figure 7.3A). These results were as expected based on the hypothesis discussed in 7.3.1.1. Overall protoplast yields were similar to those reported in table 7.1, however yields must be related to the initial quantity of mycelial biomass used. The actual mycelial biomass, although not quantitatively measured in either experiment, was probably much less using the method described in this second experiment. This would suggest that this method is more effective than that described in section 7.3.1.1. Reasons for this could be due to greater maceration using the Waring blender or the use of younger mycelia. Protoplast regeneration was slightly greater using 48 h cultures, however the difference was not significant (figure 7.3B). These results indicate that the modified experimental method is better in the production of protoplasts and was therefore used in subsequent studies.

Table 7.2. Effect of culture age and enzyme exposure time on protoplast yield and regeneration of *C. marmorata*. Values in bold text indicate highest level of protoplast produced.

	Culture age			
	24 hour		48 hour	
Incubation time (min)	Yield (ml^{-1})	Regeneration (%)	Yield (ml^{-1})	Regeneration (%)
15	2.4×10^6	8	0.7×10^6	9
30	3.8×10^6	11	1.2×10^6	11
45	**3.0×10^6**	13	1.9×10^6	13
60	2.7×10^6	14	**2.2×10^6**	16
75	2.6×10^6	14	1.5×10^6	15
90	2.7×10^6	12	1.3×10^6	14

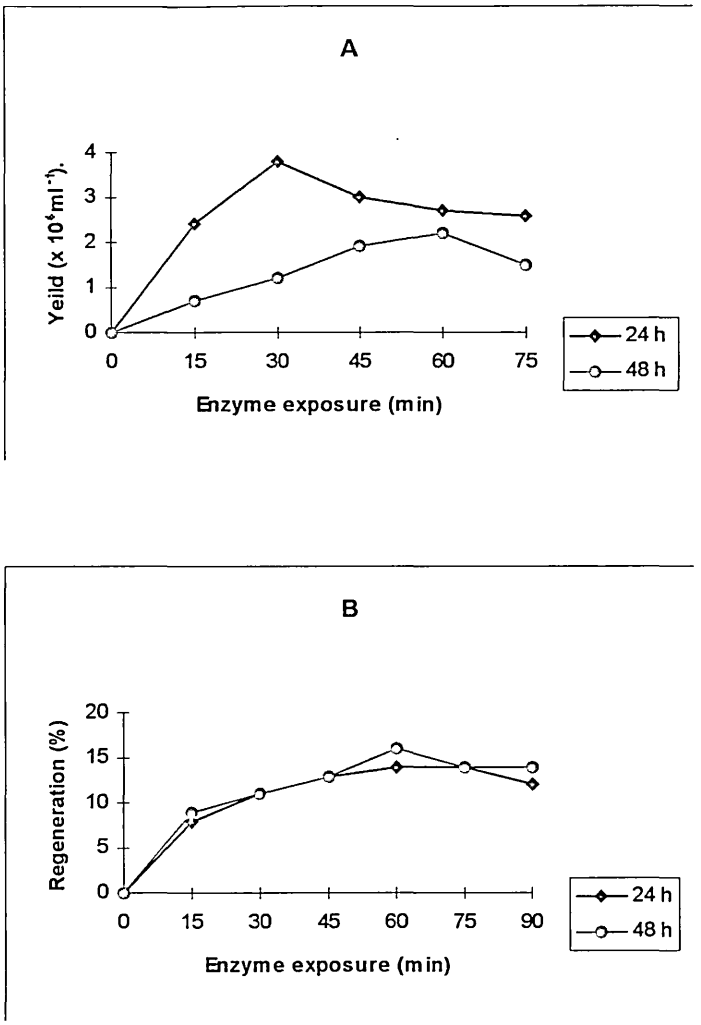


Figure 7.3. Effect of culture age and enzyme exposure time on (A) protoplast yield, and (B) protoplast regeneration of *C. marmorata.*

7.3.1.3. Effect of buffer pH on protoplast yield

Protoplast yield and regeneration was greatest at pH 5.5 and both parameters decreased slightly as buffer pH was increased (table 7.3). The effect of buffer pH on protoplast yield and regeneration was similar to results reported by Rui &

Morrell (1993). Studies of the effect of pH on protoplast production by *Penicillium chrysogenum* have also shown optimal protoplast yield around pH values of 5.5, however little evidence of mycelial digestion was found at pH values below 4.0, and above pH 8.0, and subsequently protoplasts lysed on emergence (Eyssen, 1977). The effects of pH on protoplast yield are most probably due to deconfiguration of the operational structure of the lytic enzymes. Typical effects of pH on enzymes include, enzyme - substrate binding, catalytic activity of the enzyme, and the variation of protein structure (Voet & Voet, 1990). In conclusion, the results of this investigation suggest that an osmotic stabilisation buffer pH of 5.5 is optimal for protoplast production and regeneration.

Table 7.3. Effect of pH on *C. marmorata* protoplast yield and regeneration.

pH	Protoplast Yield	Regeneration (%)
5.5	**1.3 x 10^6**	**14**
6.0	1.2 x 10^6	12
7.0	0.9 x 10^6	11

7.3.1.4. Effect of osmotic stabiliser concentration on protoplast release

Results of the effect of osmotic stabiliser on protoplast yield and regeneration are presented in table 7.4. In general, protoplast yields were significantly higher than previously reported at all three concentrations. In particular, protoplast yield was greatest with 1 M and lowest with 0.5 M mannitol. Regeneration of protoplasts was not significantly different from previous studies reported in sections 7.3.1.1.

– 7.3.1.3. These results indicate that osmotic buffer concentration was important for the production of *C. marmorata* protoplasts allowing the retention of their structural integrity.

Table 7.4. Effect of osmotic stabiliser concentration on *C. marmorata* protoplast yield and regeneration.

Mannitol concentration	Yield (ml^{-1})	Regeneration (%)
0.50 M	1.6×10^7	17
0.75 M	4.3×10^7	17
1.00 M	**9.3×10^7**	**19**

7.3.2. Protoplast sensitivity to the antibiotic hygromycin B

Growth of *C. marmorata* protoplasts was completely inhibited at a hygromycin concentration of 50 μg ml^{-1} (table 7.5), however, some reduction in the growth of *C. marmorata* was observed at 25 μg ml^{-1}. Hygromycin B is an aminoglycoside antibiotic commonly used as a selective agent for the inhibition of basidiomycete fungal growth. Its mode of action involves the mistranslation of mRNA, therefore inhibiting protein synthesis (Gale, 1981).

Table 7.5. Effect of hygromycin B on the growth of *C. marmorata* protoplasts (0 = no growth, 3 = normal growth in absence of hygromycin B).

Hygromycin concentration (μg ml^{-1})	Protoplast growth
0	3
25	2
50	0
75	0

7.3.3. Fungal Transformation

7.3.3.1. Fungal transformation 1

The growth of *C. marmorata* protoplasts was inhibited completely by a concentration of 50 μg ml^{-1} hygromycin B (in agreement with results presented in table 7.5) indicating that selection for hygromycin resistant colonies, via integration and expression of the *hyg* gene, should be possible above this concentration. However, no hygromycin resistant colonies were found in any of the transformation studies attempted. Furthermore, green fluorescence was not detected in any of the colonies formed during transformation with pTEFEGFP. This transformation experiment was performed in triplicate, however no transformants were found in any of the investigations. These results suggest that either the vector DNA was not integrated into the fungal genome or that on integration the vector DNA was not expressed. Although not attempted, analysis of gene integration into the fungal genome could be possible using southern blotting techniques using a radio-labelled hybridisation probe constructed from the plasmid DNA (Sambrook *et al.*, 1989). This method also allows

characterisation of the number of copies and sites of gene integration. Expression of heterologous genes in basidiomycete fungi has been shown to be problematic (Schuren & Wessels, 1998; P. Birch, Personal Communication). Two main causes may be the inability of host species to use regulatory sequences not derived from basidiomycetes, and/or recognition and inactivation of heterologous genes (normally from prokaryotic origin) (Schuren & Wessels, 1998). Furthermore, several studies have shown that the regulatory sequences from ascomycetes do not operate in basidiomycetes (Casselton & Herce, 1989; Moonibrook, *et al.*, 1990; Schurn & Wessels, 1998), suggesting a lack of sequence similarity between different taxonomic groups of fungi. Therefore, a possible explanation for the failure of *C. marmorata* to express the GFP gene may be due to the use of a deuteromycete gene promoter and an ascomycete terminator sequence fused to the GFP gene.

The disappointing results obtained during these transformations lead to the redesign of the experimental procedure. Due to the possible lack of sequence similarity in the GFP vector it was decided to use only the pPHT vector in subsequent studies. It was hoped that the presence of basidiomycete regulatory sequences could share enough similarity for transformation to be successful. Also, the transformation reaction conditions were altered by increasing the concentration of calcium chloride from 25 mM to 40 mM according to studies by Alic, *et al.*, (1989).

Table 7.6. Results of transformation study 1 (0 = no growth, 3 = normal growth in absence of hygromycin B).

	Hygromycin concentration (μg ml^{-1})				
Sample	**0**	**25**	**50**	**75**	**100**
Control	3	1	0	0	0
pPHT1	3	1	0	0	0
pPHT1 + pTEFEGFP (circular)	3	1	0	0	0
pPHT1 + pTEFEGFP (linear)	3	1	0	0	0

7.3.3.2. Fungal transformation 2

As stated previously (Table 7.5), protoplast regeneration was completely inhibited at a hygromycin B concentration of 50 μg ml^{-1}. Although, the PEG, $CaCl_2$ and plasmid concentrations were increased in this study, no hygromycin resistant *C. marmorata* colonies were produced (Table 7.6 and 7.7). The exact fusogenic mechanism of PEG on the cell membrane is still unresolved. However, studies carried out by Knutton (1979) and Ahkong *et al* (1975) on PEG treated erythrocyte membranes indicated that protein particles are translocated leaving lipid rich regions at the site of close membrane contact. Reorganisation of the lipid – lipid interface in the presence of calcium ions results in the fusion of membranes (Peberdy, 1989). Due to the lack of transformants in either study it was not possible to assess the attempted optimisation of the transformation procedure in this set of experiments. Therefore, the results given in this experiment could suggest that there is too little sequence homology between the

regulatory regions of *C. cinereus* and *C. marmorata* for successful plasmid integration.

Table 7.7. Results of transformation study 2 (0 = no growth, 3 = normal growth in absence of hygromycin B).

	Hygromycin concentration (μg ml^{-1})				
Sample	**0**	**25**	**50**	**75**	**100**
Control	3	1	0	0	0
pPHT1 (2 μg)	3	1	0	0	0
pPHT1 (4 μg)	3	1	0	0	0

7.4. CONCLUSIONS

Although the transformation of *C. marmorata* was not successful, it was possible to produce protoplasts from this species. At present, there have been no published reports of protoplast production in *C. marmorata*. As suggested above, the success of the genetic transformation study was most probably hampered by a lack of genomic information of this species. In general, most successful fungal genetic transformations have been made in well-characterised 'model' species, such as *Aspergillus niger, Phytophthora infestans,* and *Ustilago maydis*, where the promoter sequences contained within the DNA vectors have a high degree of homology to the host species. These species (and others not mentioned) have received considerable investigation due to the economic loss incurred by their action as plant pathogens. Indeed, monitoring the distribution of fungal biomass in planta and understanding the mechanisms of host-pathogen interactions have been significantly advanced with the application of GFP technologies (Lorang *et al.* 2001).

The future of successful genetic transformation in fungi will undoubtedly be advanced as the complete genetic sequences of several filamentous fungi (e.g. *Ustilago maydis, Phytophthora*, and *A. nidulans*) are elucidated through genome projects currently in progress (Rogers, 2000; Yoder and Turgeon, 2000). It is conceivable that such information will lead to the characterisation of promoter sequences for every gene in such species and potentially enable any gene to be labelled using GFP. Furthermore, this genomic information in combination with improved methods of introduction of foreign DNA into fungi should also increase the success of GFP technologies in fungal biology.

CHAPTER 8. GENERAL DISCUSSION

8.1. AN OVERVIEW OF THE PROJECT FINDINGS

The aim of this chapter is to discuss the main findings of the project along with their relative merits and limitations. In addition, a discussion of potential areas for further research is described. This thesis aimed to determine the important factors influencing the community dynamics of selected wood decay fungi isolated from a local historic ship, the Frigate Unicorn.

The importance of biotic interactions, especially antagonistic interactions, in determining the structure and development of fungal communities in woody, submerged and soil environments has long been established (Rayner & Boddy, 1988; Stahl & Christensen, 1992; Boddy, 2000). However, most laboratory based studies have been performed at relatively small spatial scales (often confrontation of two mycelial individuals on a common nutrient source) and often do not account for the variety of responses a fungal individual can exhibit when subjected to the same experimental conditions. Relating the outcome of such interaction studies to community development in the natural environment is therefore complex especially in terms of temporal dynamics and biological context. Therefore, the predictive capacity of theoretical models based on such data is limited. In an attempt to address these problems, the influence of spatial scale and heterogeneity in interaction outcome between replicates were key factors investigated in this thesis.

An experimental microcosm system was developed which would permit the controlled study of the interspecific interactions that are prevalent during the development of fungal communities (Chapter 3). The microcosm consisted of square tiles of nutrient agar that could be inoculated and prescribed in a variety of spatial patterns. The system also allowed the quantification of interaction outcome by determining the occupancy of each fungal species within each quarter tile as the interaction progressed by a destructive harvest and re-isolation technique. In Chapter 3, the outcome and reproducibility of pairwise confrontations between the three selected fungi was examined and the important experimental factors influencing their interaction outcomes were determined. It was demonstrated that the interaction outcomes between replicates were often not reproducible and displayed both spatial and temporal heterogeneity. Furthermore, the variation in outcome was more prevalent during interactions incubated at ambient temperature, indicating that microclimate fluctuations influence heterogeneity. In general, *C. marmorata* replaced *P. variotii* and *P. placenta*^{1} showed a deadlock interaction with *P. variotii*. However, the interaction between *P. placenta*^{1} and *C. marmorata* displayed various outcomes depending on experiment with *P. placenta*^{1} replacing or deadlocking with *C. marmorata* in preliminary experiments and *C. marmorata* replacing or deadlocking with *P. placenta*^{1}, during later investigations.

It was important to determine the important experimental factors influencing interaction outcome for the development of subsequent larger scale experiments. It was found using the stepwise logistic regression model that the presence of a species on outcome of the interactions was influenced by four factors. Ranked in order of importance, these factors were; (1) initial presence within a tile, (2) species combination, (3) longest sampling period, and (4) inoculation mode.

Using the microcosm system developed in Chapter 3, the effects of scaling on two species interactions was investigated (Chapter 4). This was considered a logical first step in increasing the complexity of the experimental system. In general, it was found that the final interaction outcomes of the large-scale two species interactions were in agreement with the outcomes of the relevant small-scale binary tile interactions. However, further investigation revealed that the temporal dynamics of the large-scale interactions were dependent on patch size. For example, the rate of invasion of *P. variotii* tiles and eventual replacement by *C. marmorata* was increased as patch size decreased. These findings suggest that the temporal dynamics are influenced by some factor other than independent nearest neighbour interactions. Indeed, the application of mathematical based modelling techniques showed that non-independent local interactions are important in determining the dynamics at the community-scale. It is proposed that processes such as anastomosis of genetically similar individuals may lead to translocation of resources from sites of low combative stress to interaction fronts where antagonistic or defensive responses increase metabolic demand and thus effect the temporal dynamics of the system. It is important to realise that quantification of such scaling effects can only be made at the level of the community and therefore the extrapolation of temporal dynamics from experiments below this level is not possible.

Although prediction of the temporal dynamics of the large-scale two species interactions were not possible from the small-scale, binary tile, confrontations it was possible to predict their final interaction outcomes. Final interaction outcomes of pairwise confrontations are still commonly used to derive combative hierarchy information used in predictive *in-situ* fungal ecology. However, during multi-species interactions, problems may arise in the

application of combative hierarchies in predicting the development of natural communities due to intransitivity and interaction modification effects (Boddy, 2000). Indeed, this was the case when the complexity of the experimental system was increased to investigate the development of all three fungal species within one interaction arena (Chapter 5). It was found that the final interaction outcomes of the three species interactions could not be extrapolated from the relevant binary tile interactions. This finding has significant consequences with respect to the use of combative hierarchy information in the prediction of fungal community development. Indeed, it should be stressed that the behaviour of two fungal individuals is specific to that pairing combination, and that the addition of a third species influence's the overall interactive functioning of the system. Thus, it can be concluded that the reductionist (bottom – up) approach used in these studies is not appropriate for the prediction of the overall behaviour of a multi-species system. This finding suggests that an alternative approach be taken to investigate the behaviour of such multi-species interactions. Such approaches could involve studies performed at a community level and/or include some features of ecological strategy and / or fundamental and realised niche. Such lines of investigation would therefore enable the incorporation of emergent behaviours that may not be apparent during small-scale binary interactions.

Indeed, consideration of the results of the three species interactions at the level of the community showed that spatial pattern was found to be a key determinant in the outcome of the interactions. The results showed that different starting configurations, even with consummate proportion of inocula, influence the outcome and reproducibility of the interactions. The novel application of principle component analysis to describe the complex spatial and temporal data revealed that the outcomes were neither random nor fully deterministic suggesting evidence of stochasticity in interaction outcome. It was found,

however, that the 3 x 3 interactions displayed more consistent outcomes compared to the larger-scale 6 x 6 interactions. This result may support findings shown in Chapter 4 that suggest spatial scale influences the temporal dynamics of the system. Therefore, due to the smaller spatial scale of the 3 x 3 tessellations a more stable outcome was achieved faster than the larger 6 x 6 tessellations.

A major advantage of the tile-based microcosm developed in this project was that the results of the interaction studies could be used to inform 'rules' for a cellular automaton (CA) computer-modelling program. This was mainly possible due to the method of harvest used, which essentially produced spatially discrete data. Furthermore, CA models are stochastic and therefore able to include the variability displayed in interaction outcome between replicates during the binary tile interactions. However, as was shown with the application of this modelling technique to predict the dynamics of the large-scale two species interactions, the predictions made are only as good as the data used to form its operational rules. Therefore, it was not possible to predict the effect of emergent behaviours on the interaction dynamics shown in the large-scale interactions because the binary tile confrontations used to calibrate the model could only provide data relating to independent nearest neighbour interactions.

Although the data produced by the destructive harvest of the tiles within the microcosms can be viewed suitable for the application to discrete modelling techniques such as cellular automaton, this method is also a limitation of the tile-based microcosm. This is because destructive sampling of the tiles inherently demands that large numbers of replicates are required for temporal monitoring of the interactions and that the development of individual tiles cannot be followed exactly. Thus, the experimental work developed in Chapter 7

aimed to develop a method for continuous, non-destructive analysis of the fungal community development. Application of such a method would also mean that the tile-based system could be avoided thus making the system more realistic. The conceptual framework proposed was to label one of the three fungi used in the interaction studies with green fluorescent protein via genetic transformation. Labelling of this species would enable the precise quantification of various characteristics of its growth (e.g. area of individual patch, degree of connectedness, area to perimeter ratio and other measures of shape) within a microcosm-based system. However, the results of these experiments presented in Chapter 7 indicate that the genetic transformation was not achieved. It would be realistic to assume that this was probably due to a paucity of information regarding the genetics of *C. marmorata*. The introduction and expression of exogenous DNA in basidiomycete fungi is still in its infancy and successful transformations tend to be limited to well-characterised model or economic and biotechnologically important species such as *Phanerochaete chrysosporium, Schizophyllum commune* and *Coprinus cinereus*. However, the conceptual approach developed has received some attention with other investigators interested in the behaviour of fungi in natural environments such as soil. In particular, Bae and Knudson (2000) successfully applied GFP labeling of *Trichoderma harzianum* to monitor its growth and activity in soils. Therefore, it can be envisioned that with the availability of suitable GFP labelled fungal species the study of fungal communities could be significantly advanced.

The variety of growth responses capable by fungi is another important consideration during investigation of their community dynamics. During the interaction studies presented in Chapter 4, results indicated that the behaviour of *P. placenta* was different in both

morphology and combative ability compared to the results of the small-scale binary tile interactions. The investigation of this phenomenon showed that a morphologically distinct form of *P. placenta (P. placenta*^{2}*)* had developed. It was difficult to determine the cause of this event and it was proposed that continued storage and subculture *in-vitro* may have caused the 'switch' in behaviour. It was found that growth of *P. placenta*^{2} on its natural substrate, wood, and subsequent re-isolation after 2 months, re-established a similar level of combative ability against *C. marmorata* compared its original form. This finding may be considered as evidence supporting hypotheses regarding the loss of morphological and physiological attributes through continued storage and/or growth in artificial culture.

8.2. FUTURE RESEARCH

The experimental system presented in this thesis provided a novel approach to studying the interspecific interactions of fungi in spatially heterogeneous systems. There is a growing awareness that spatial aspects of ecosystems, while currently impeding reliable quantitative understanding, are a key to promoting the persistence of diversity in such systems (Tilman, 1994; Rayner, 1996). Therefore, the development of suitable theoretical and experimental frameworks to investigate the role of spatial heterogeneity on community dynamics is an important area for future research. The findings presented in this thesis clearly show that quantification of the dynamics of fungal communities is not possible outwith the scale of the community. Thus, future experimental investigation should be conducted at the spatial scale of interest. Such an approach would enable a more realistic, continuous system to be investigated rather than the artificial discrete nature of the tile interaction system. However, a continuous spatial domain would also impose further complexity to the system in terms

of quantification of the occurrence of species (as the various species would no longer be confined to specific tile domains). Therefore, the development of a continuous monitoring system would also be a requisite to accurately map the occurrence and possibly the activity of various interacting fungal individuals. The conceptual framework developed in Chapter 7, involving the use of GFP-labeling and image analysis has the potential to enable continuous monitoring when such species become available. Although essentially discontinuous, other molecular based technologies such as quantitative polymerase chain reaction and DNA micro-array technologies may have the potential for application to the quantitative monitoring distribution and activity of fungal species within the natural environment.

The modelling of fungal communities has important relevance to the application and management of biocontrol and bioremediation strategies, predicting community dynamics with climate change and assessing the effects of environmental management of communtiy dynamics and hence decompostion rates and diversity. The use of laboratory based experimental systems may provide important information on the effectiveness potential biocontrol or bioremediation agents before full-scale application in the natural environment.

REFERENCES

Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A. (1975). Mechanisms of cell fusion. *Nature*, **253**, 194-195.

Ainsworth, A. M., Beeching, J. R., Broxholme, S. J., Hunt, B. A., Rayner, A. D. M., and Scard, P. T. (1992). Complex outcome of reciprocal exchange of nuclear DNA between two members of the basidiomycete genus *Stereum*. *Journal of General Microbiology*, **138**, 1147-1157.

Aleksenko, A. Y. and Clutterbuck, A. J. (1995). Recombinational stability of replicating plasmids in *Aspergillus nidulans* during transformation, vegetative growth and sexual reproduction. *Current Genetics*, **28**, 87-93.

Alexopopoulos, G. C., Mimms, C. W. and Blackwell, M. (1996) *Introductory Mycology*. John Wiley & Sons, New York.

Alic, M., Kornegay, J.R., Pribnow, D. and Gold, M.H. (1989). Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology*, **55**, 406-411.

Allen, T. F. H., King, A. W., Milne, B. T., Johnson, A., and Turner, S. (1993). The problem of scale in ecology. *Evolutionary Trends in Plants*, **7**, 3-8.

Austin, B., Hall, R. M., Tyler, B. M. (1990). Optimized vectors and selection for transformation of *Neurospora crassa* and *Aspergillus nidulans* to bleomycin and phleomycin resistance. *Gene*, **93**, 157-162.

Bae, Y. S. and Knudson, G. R. (2000) Cotransformation of *Trichoderma harzianum* with beta-glucuronidase and green fluorescent protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. *Applied and Environmental Microbiology*, **66**, 810.

Barman, T. E. (1969). *Enyzme Handbook.* Volume I & II. Springer-Verlag, Berlin Heidelberg. New York.

Barrow, J. D. (1999). *Between inner and outer space*. Oxford University Press, Oxford.

Billick, I. and Case, T. J. (1994). Higher-order interactions in ecological communities – what are they and how can they be detected. *Ecology*, **75**, 1529-1543.

Birch, P. R. J., Sims, P. F. G. and Broda, P. (1998). A reporter system for analysis of regulatable promoter functions in the basidiomycete fungus *Phanerochaete chrysosporium*. *Journal of applied microbiology*, **85**, 417-424.

Boddy, L. (1993). Saprotrophic cord forming fungi: warfare strategies and other ecological aspects. *Mycological Research*, **97**, 641-655.

Boddy, L. (2000). Interspecific combative interactions between wood-decaying basidiomycetes. *FEMS Microbiology Ecology*, **31**, 185-194.

Boddy, L., Gibbon, O. M., and Grundy, M. A. (1985). Ecology of *Daldinia concentrica*: effect of abiotic variables on mycelial extension and interspecific interactions. *Transactions of the British Mycological Society*, **85**, 201-211.

Bown, J., Sturrock, C. J., Samson, W.B., Staines, H.J., Palfreyman, J.W., White, N.A., Ritz, K. and Crawford, J. (1999). Evidence for emergent behaviour in the community-scale dynamics of a fungal microcosm. *Proceedings of the Royal Society London* B, **266**, 1947-1952.

Bown, J. L. (2000). Issues of scale in individual-based models: applications in fungal and plant community dynamics. Ph. D. Thesis. University of Abertay Dundee, Dundee.

Bruce, A., Austin, W. J. and King, B. (1984). Control and growth of *Lentinus lepideus* by volatiles from *Trichoderma*. *Transactions of the British Mycological Society,* **82**, 423-428.

Bruns, T. D. and Gardes, M. (1993). Molecular tools for the identification of ectomycorrhizal fungi – taxon-specific oligonucleotide probes for suilloid fungi. *Molecular Ecology*, **2**, 233-242.

Buller,A. H. R. (1933). *Researches on Fungi*, Vol. 5, Longman, London.

Carlile, M. J. and Watkinson, S. C. (1994). *The Fungi*. Academic Press, London.

Carreiro, M. M. and Koske, R. E. (1992). Effect of temperature on decomposition and development of micro-fungal communities in leaf litter microcosms. *Canadian Journal of Botany*, **70**, 2177-2183.

Casselton, L. A. and Herce, A. D. (1989). Heterologous gene expression in the basidiomycete fungus *Coprinus cinereus*. *Current Genetics*, **16**, 35-40.

Chapela, I. and Boddy, L. (1988a). Fungal colonization of attached beech branches. I. Early stages of development of fungal communities. *New Phytologist*, **110**, 39-45.

Chapela, I. and Boddy, L. (1988b). Fungal colonization of attached beech branches. II. Spatial and temporal organization of communities arising from latent invaders in bark and functional sapwood, under different moisture regimes. *New Phytologist*, **110**, 47-57.

Chapela, I., Boddy, L. & Rayner, A.D.M. (1988). Structure and development of fungal communities in beech logs four and a half years after felling. FEMS Microbiology Ecology, **53**, 59-70.

Charfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, **263**, 802-805.

Christensen, M. (1989). A view of fungal ecology. *Mycologia*, **81**, 1-19.

Coates, D. and Rayner, A. D. M. (1984). Fungal population and community development in beech logs. III. Spatial dynamics, interactions and strategies. *New Phytologist*, **101**, 183-198.

Coates, D. and Rayner, A.D.M. (1985). Fungal population structure and community development in cut beech logs. III. Spatial dynamics, interactions and strategies. *New Phytologist*, **101**, 183-198.

Cooke, R.C. and Rayner, A.D.M. (1984). *Ecology of Saprotrophic fungi.* Longman, London.

Cooke, R. C. and Whipps, J. H. (1993). *Ecophysiology of Fungi.* Blackwell, Oxford.

Cormack, B. P., Bertram, G., Egerton, M., Gow, N. A. R., Falkow, S., and Brown, A. J. P. (1997). Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology,* **143**, 303-311.

Cormack, B. (1998). Green fluorescent protein as a reporter of transcription and protein localization in fungi. *Current opinion in microbiology*, **4**, 406-410.

Deacon, J. W. (2000). *Modern Mycology*. 3rd Ed. Blackwell, Oxford.

Dewey, F. M. (1992). Detection of plant invading fungi. In: *Techniques for the rapid detection of plant pathogens.* (Ed. Duncan, J. M. and Torrance, L.). Blackwell, Oxford.

Dowson, C. G., Rayner, A. D. M. and Boddy, L. (1986). Outgrowth patterns of mycelial cord-forming basidiomycetes from and between woody resource units in soil. *Journal of General Microbiology*, **121**, 203-211.

Dowson, C. G., Rayner, A. D. M. and Boddy, L. (1988). The form and outcome of mycelial interactions involving cord-forming decomposer basidiomycetes in homogeneous and heterogeneous environments. *New Phytologist*, **109**, 423-432.

Dumas, B., Centis, S., Sarrazin, N., EsquerreTugaye, M. T. (1999). Use of green fluorescent protein to detect expression of an endopolygalacturonase gene of *Colletotrichum lindemuthianum* during bean infection. *Applied and Environmental Microbiology*, **65**, 1769-1771.

Egger, K. N. (1992). Analysis of fungal population structure using molecular techniques. In: *The Fungal Community: Its Organisation and Role in the Ecosystem.* (Ed. Carroll, G. C. and Wicklow, D. T.). pp 193-208. Marcel Dekker, New York.

Eyssen, H. (1977). Formation of protoplasts of *Penicillium chrysogenum* and some other filamentous fungi. *Agricultura*, **25**, 21-44.

Findlay, G. W. D. and Levy, J. F. (1969). Scanning electron microscopy as an aid to the study of wood anatomy and decay. *Journal of the Institute of Wood Science*, **23**, 57-63.

Fernandez-Abalos, J. M., Fox, H., Pitt, C., Wells, B., Doonan, J. H. (1998). Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein

localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. *Molecular Microbiology*, **27**, 121-130.

Frankland, J.C. (1998). Fungal succession – unravelling the unpredictable. *Mycological Research*, **102**, 1-15.

Gale, E. F. (1981) *Molecular basis of antibiotic action*. 2nd Edition. John Wiley & Sons, New York.

Gardes, M. and Bruns, T. D. (1993). ITS Primers with enhanced specificity for basidiomycetes – applications on mycorrhizae and rusts. *Molecular Ecology*, **2**, 113-118.

Genstat 5 Committee. (1993). *Genstat 5 Release 3 Reference Manual*. Oxford, Clarendon Press.

Goodell, B. S. and Jellison, J. (1986). Detection of a brown rot fungus using serological assays. *International Research Group on Wood Preservation,* Document No. IRG/WP/1305.

Griffith, G. S. and Boddy, L. (1991). Fungal decomposition of attached angiosperm twigs. IV. Effect of water potential on interactions between fungi on agar and wood. . *New Phytologist*, **177**, 633-641.

Gull, K. (1978). Form and function of septa in filamentous fungi. In: *The Filamentous Fungi: Developmental Mycology*. (Ed. Berry, D. R. and Smith, J. E.). Arnold, U. K.

Gurr, E. (1965). *The Rational Use of Dyes in Biology*, Leonard Hill, London.

Halley, J. M., Comins, H. N., Lawton, J. H. and Hassell, M. P. (1994). Competition, succession and pattern in fungal communities: towards a cellular automaton model. *OIKOS*, **70**, 435-442.

Halley, J. M., Robinson, C. H., Comins, H. N. and Dighton, J. (1996). Predicting straw decomposition by a 4-species fungal community - A cellular-automaton model. *Journal of Applied Ecology*. **33**, 493 - 507.

Harold, F. M. (1997). How hyphae grow: Morphogenesis explained? *Protoplasma*, **197**, 137-147.

Hawksworth, D. L. (1991). The dimension of biodiversity: magnitude, significance, and conservation. *Mycological Research*, **95**, 641-655.

Hawksworth, D. L. (2000). Mycological research in the new millennium. *Mycological Research*, **104**, 1-1.

Holland, J. H. (2000). Emergence: From chaos to order. Oxford University Press.

Holmer, L & Stenlid, J. (1993). The importance of inoculum size for the competitive ability of wood decomposing fungi. *FEMS Microbiology Ecology*, **12**, 169-176.

Hooley, P. (1993). Genetic engineering of fungi. In. *Strategies for engineering organisms.* Ed. Bruns, A. T. H. Butterworth-Heinemann, Oxford.

Horsthemke, W. and Lefever, R. (1984). *Noise Induced Transitions: Theory and Applications in Physics, Chemistry and Biology.* Berlin: Springer Verlag.

Hunsley, D. and Gooday, G. W. (1974). The structure and development of septa in *Neurospora crassa. Protoplasma*, **82**, 125-146.

Hunt, R. S. and Cobb, F. W. (1971). Selective medium for the isolation of wood-rotting basidiomycetes. *Canadian Journal of Botany*, **49**, 2064-2065.

Hynes, M. J. (1996). Genetic transformation of filamentous fungi. *Journal of Genetics*, **75**, 297-311.

Johnson, F. H. and Shimomura, O. (1978). *Methods in Enzymology*, **57**, 271-291.

Kamper, J., Reichmann, M., Romeis, T., Bolker, M. and Kahmann. R. (1995). Multiallelic recognition: non-self dependent dimerization of the *bE* and *bW* homeodomain proteins in *Ustilago maydis. Cell*, **81**, 73-83.

Knutton, S. (1979). *Journal of Cell Science*, **80**, 64-

Kotliar, N. B. and Wiens, J. A. (1990). Multiple scales of patchiness and patch structure – a hierarchical framework for the study of heterogeneity. *OIKIOS*, **59**, 253-260.

Levene, H. (1960). *Contributions to Probability and Statistics*. Pp. 278-292. Stanford University Press, California.

Levin, S. A. (1992). The problem of pattern and scale in ecology. *Ecology*, **73**, 1943-1967.

Li, L. T. and Kaplan, J. (1997). Characterization of two homologous yeast genes that encode mitochondrial iron transporters. *Journal of Biological Chemistry*, **272**, 28485-28493.

Long, T. J. and Alben, J. O. (1978). Preliminary studies of mushroom tyrosinase. *Mushroom Science*, 7, 69-79.

Lorang, J. M., Tuori, R. P., Martinez, J. P., Sawyer, T. L., Redman, R. S. Rollins, J. A., Wolpert, T. J., Johnson, K. B., Rodriguez, R. J., Dickman, M. B. and Ciuffetti, L. M. (2001). Green fluorescent protein is lighting up fungal biology. *Applied and Environmental Microbiology*, **67**, 1987-1994.

Low, G. A. (2000). An investigation of selected effects of environment on the dry rot fungus *Serpula lacrymans*. Ph. D. Thesis, University of Abertay Dundee, Dundee.

Magan, N and Lacey, J. (1984). Effect of water activity, temperature and substrate on interactions between field and storage fungi. *Transactions of the British Mycological Society*, **82**, 83-93.

Marmeisse, R., Gay, G., Debaud, J. and Casselton, L. A. (1992). Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. *Current Genetics*, **22**, 41-45.

Metzler, D. E. (1977). *Biochemistry*. Iowa State University Press, Iowa.

Momany, M. and Hamer, J. E. (1997). The *Aspergillus nidulans* septin encoding gene, aspB, is essential for growth. *Fungal Genetics and Biology*, **21**, 92-100.

Moonibrook, H., Kuipers, A. G. J., Sietsma, J. H., Punt, P. J., and Wessels, J. G. H. (1990). Introduction of hygromycin B resistance into *Schizophyllum commune*: preferential methylation of donor DNA. *Molecular and General Genetics*, **222**, 41-48.

Palfreyman, J. W. P., Bruce, A., Button, D., Glancy, H., Vigrow, A. and King, B. (1987). Immunological methods for the detection and characterization of wood decay basidiomycetes. In. *Biodeterioration* 7. (Ed. Houghton, D. R., Smith, R. N. and Eggins, H. O. W.) pp. 709-713. Cambridge, U. K. Elsevier Science Publisers Ltd.

Peberdy, J. F. (1989). Fungi without coats – protoplasts as tools for mycological research. *Mycological Research*, **93**, 1-20.

Pugh, G. J. F. (1980). Strategies in fungal ecology. *Transactions of the British Mycological Society*, **75** (1), 1-14.

Pugh, G. J. F. and Boddy, L. (1988). A view of disturbance and life strategies in fungi. *Proceedings of the Royal Society of Edinburgh*, **94B**, 3-11.

Rayner, A.D.M. (1986). Mycelial interactions – genetic aspects. In. *Natural Antimicrobial systems*. (Ed. Rhodes-Roberts, M. E., Gould, G. W. Charnley, A. K. Cooper, R. M. and Board. R. G.) pp. 277-296. Bath University Press, Bath.

Rayner A.D.M. (1991). Conflicting flows – the dynamics of mycelial territoriality. *McIlvainea*, **10**, 24-35.

Rayner, A.D.M. (1994). Pattern-generating processes in fungal communities. In: *Beyond the biomass: Compositional and functional analysis of soil microbial communities* (Eds. K. Ritz, J. Dighton & K.E. Giller). John Wiley & sons, London.

Rayner, A.D.M. (1996). Interconnectedness and individualism in fungal mycelia. In: A Century of Mycology (Ed. Sutton, B.C.). pp. 193 – 232.

Rayner, A.D.M. and Todd, N.K. (1979). Population and community structure and dynamics of fungi in decaying wood. *Advances in Botanical Research*, 7, 333-420.

Rayner A.D.M. and Webber, J. F. (1984). Interspecific mycelial interactions – An Overview. In: *The Ecology and Physiology of the Fungal Mycelium*. (Ed. Jennings, D. H. and Rayner, A. D. M.) Cambridge University Press, Cambridge.

Rayner, A.D.M., Watling, R. and Frankland, J. C. (1985). Resource relations – an overview. In. *Developmental Biology of Higher Fungi.* (Ed. Moore, D., Casselton, L. A., Wood, D. A. and Frankland, J. C.) pp. 1-40. Cambridge University Press, Cambridge.

Rayner A.D.M. and Coates, D. (1987).Regulation of mycelial organization and responses. In. *Evolutionary Biology of the Fungi.* (Ed. Rayner A.D.M., Brasier, C. M. and Moore, D.) Cambridge University Press, Cambridge.

Rayner, A.D.M. and Boddy, L. (1988). *Fungal Decomposition of Wood: Its Biology and Ecology.* New York: John Wiley.

Rayner A.D.M., Griffith, G. S. and Wildman, H. G. (1994). Differential insulation and the generation of mycelial patterns. In. *Shape and Form in Plants and Fungi.* (Ed. Ingram, D. S.) pp. 293-312. Academic Press, London.

Rayner, A.D.M., Griffith, G.S. and Ainsworth, A.M. (1995). Mycelial interconnectedness. In: *The growing fungus* (Eds. Gow, N.A.R. and Gadd, G.M.). pp. 21-37. Chapman and Hall, London.

Riechle, R. E. and Alexander, J. V. (1965). Multiperforate septa, Wororin bodies and septal plugs in *Fusarium. Journal of Cell Biology*, **24**, 489-496.

Ritz, K. (1995). Growth responses of some soil fungi to spatially heterogeneous nutrients. *FEMS Microbiology Ecology,* **16**, 269-280.

Ritz, K. and Crawford, J. W. (1999). Colony development in nutritionally heterogeneous environments. In: *The Fungal Colony.* (Ed. Gow, N. A. R., Robson, G. D. and Gadd, G. M.) pp. 49-74. Cambridge University Press, Cambridge.

Robinson, C.H.; Dighton, J. & Frankland, J.C. (1993). Resource capture by interacting fungal colonizers of straw. *Mycological Research,* **97**, 547-558.

Robson, G. (1999). Hyphal cell biology. In. *Molecular Fungal Biology.* (Ed. Oliver, R. P. and Schweizer, M.). pp 156 – 170. Cambridge University Press, Cambridge.

Rogers, R. (2000). Exelixis completes genomic sequence of model fungus *Ustilago maydis. Agricultural Genomics*, **3**, 3-4.

Rui, C. and Morrell, J. J. (1993). Production of protoplasts from selected wood degrading fungi. *Wood and Fiber Science*, **25**, 61-65.

Russell, P. (1956). Selective medium for the isolation of basidiomycetes. *Nature*, **177**, 1038-1039.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning: a Laboratory Manual.* 2nd Ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Schuren, F. H. J. and Wessels, J. G. H. (1998). Expression of heterologous genes in *Schizophyllum commune* is often hampered by the formation of truncated transcripts. *Current Genetics*, **33**, 151-156.

Score, A. J., Bruce, A., King, B. and Palfreyman, J. W. (1998). The biological control of *Serpula lacrymans by Trichoderma* species. *Holzforschung*, **52**, 124-132.

Sharland, P. R., Burton, J. L. and Rayner, A. D. M. (1986). Mycelial dimorphism, interactions and pseudosclerotial plate formation in *Hymenochaete corrugata. Transactions of the British Mycological Society*, **86**, 158-163.

Sheen, J., Hwang, S., Niwa. Y., Kobayashi, H. and Galbraith, D. W. (1995). Green fluorescent protein as a new vital marker in plant cells. *Plant Journal*, **8**, 777-784.

Spellig, T., Bottin, A., and Kahmann, R. (1996). Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago mayis. Molecular and General Genetics*, **252**, 503-509.

Stahl, P.D. and Christensen, M. (1992). *In vitro* mycelial interactions among members of a soil microfungal community. Soil Biology and Biochemisty, **24**, 309-316.

Stalpers, J.A. (1978). Identification of wood-inhabiting fungi in pure culture. *Studies in Mycology,* **16**, 1-248.

Sturrock, C. J., Ritz, R. Samson, W. B., Bown, J., Staines, H. J., Palfreyman, J. W., Crawford, J. W. and White, N. A. (2002). Investigating the effects of fungal inoculum

arrangement (scale and context) on emergent community development in and agar model system. *FEMS Microbial Ecology,* **30**, 9-16.

Suelmann, R. and Fischer, R. (2000). Mitochondrial movementand morphology depend on intact actin cytoskeleton in *Aspergillus niger. Molecular Microbiology*, **30**, 831-842.

Thornewell, S. J., Peery, R. B., and Skatrud, P. L. (1995). Integrative and replicative genetic transformation of *Aureobasidium pullulans. Current Genetics*, **29**. 66-72.

Tilman, D. (1994). Competition and biodiversity in spatially structured habitats. *Ecology,* **75**, 2-16.

Tilman, D. and Kareiva, P. (1997). *The role of space in population dynamics and interspecific interactions*. Princeton University Press.

Vanden Wymelenberg, A. J., Cullen, D., Spear, R. N., Schoenike, B., and Andrews, J. H. (1997). Expression of green fluorescent protein in *Aureobasidium pullulans* and quantification on leaf surfaces. *Biotechniques*, **23**, 686-690.

Voet, D., and Voet, J. G. (1990). *Biochemistry*. Wiley, New York.

Vogt, K. A., Bloomfield, J., Ammirati, J. F. and Ammirati, S. R. (1992). Sporocarp production by basidiomycetes with emphasis on forest ecosystems. In: *The Fungal Community: Its Organisation and Role in the Ecosystem.* (Ed. Carroll, G. C. and Wicklow, D. T.) Marcel Dekker, New York.

Watkinson, S. (1999).Metabolism and hyphal differentiation in large basidiomycete colonies. In: *The Fungal Colony.* (Ed. Gow, N. A. R., Robson, G. D. and Gadd, G. M.) pp. 49-74. Cambridge University Press, Cambridge.

Weisberg, S. (1985). *Applied Linear Regression.* Wiley, New York.

Wessels, J. G. H. (1991). Fungal growth and development: a molecular perspective. In: *Frontiers in Mycology.* (Ed. Hawksworth, D. L.) CABI.

White, N.A. and Boddy, L. (1992). Differential extracellular enzyme production in colonies of *Coriolus versicolor, Phlebia radiata* and *Phlebia rufa* : effect of gaseous regime. *Journal of General Microbiology,* **138**, 2589-2598.

White, N. A., Palfreyman, J. W. and Staines, H. J. (1996). Fungal colonization of the keelson timbers of a nineteenth century wooden frigate. *Material und Organismen,* **30**, 117-131.

White, N.A., Sturrock, C., Ritz, K., Samson, W.B., Bown, J., Staines, H.J., Palfreyman, J.W. and Crawford, J. (1998). Interspecific fungal interactions in spatially heterogeneous systems. *FEMS Microbiology Ecology*, **27**, 21-32.

Wijesinghe, D. K. and Hutchings, M. J. (1997). The effects of spatial scale of environmental heterogeneity on the growth of a clonal plant: An experimenetal study with *Glechoma hederacea. Journal of Ecology*, **85**, 17-28.

Wilcox, W. W. (1964). Preparation of decayed wood for microscopical examination. *US Department of Agriculture and Forestry Service Research Paper*, FPL70.

Yoder, O. C. and Turgeon, B. G. (2000). Genomics of phytopathogenic fungi. *Phytopathology*, **90**, S109.

APPENDIX : LIST OF MANUFACTURERS

Bibby - Sterilin Ltd, Stone, Staffordshire, England, U.K.

CLONTECH Laboratories, Palo Alto, California, USA.

Merck Eurolab Ltd, Lutterworth, England, U.K.

Oxoid Ltd, Basingstoke, Hampshire, England, U.K.

Promega UK, Southampton, England, U.K.

Qiagen Ltd, Crawley, West Sussex, England, U.K.

RS components Ltd, Corby, Northants, England, U.K.

Sigma-aldrich Company Ltd, Poole, Dorset, England, U.K.

The three published articles cited below have been removed from the e-thesis due to copyright restrictions:

White, N.A., Sturrock, C., Ritz, K., Samson, W.B., Bown, J., Staines, H.J., Palfreyman, J.W. and Crawford, J. (1998). Interspecific fungal interactions in spatially heterogeneous systems. In *FEMS Microbiology Ecology,* 27, pp.21-32.

Bown, J.L., Sturrock, C.J., Samson, W.B., Staines, H.J., Palfreyman, J.W., White, N.A., Ritz, K. and Crawford, J.W. (1999). Evidence for emergent behaviour in the community-scale dynamics of a fungal microcosm. In *Proceedings of the Royal Society London B*, 266, pp.1947-1952.

Sturrock, C.J., Ritz, K., Samson, W., Bown, J.L., Staines, H.J., Palfreyman, J.W., Crawford, J.W. and White, N.A. (2001). The effects of fungal inoculum arrangement (scale and context) on emergent community development in an agar model system. In *FEMS Microbiology Ecology*, 39, pp.9-16.